Substrate Specificity of Human Nucleoside-diphosphate Kinase Revealed by Transient Kinetic Analysis

Sabine Schaertl, Manfred Konrad‡, and Michael A. Geeves§

From the Max-Planck Institut für biophysikalische Chemie, Abteilung Molekulare Genetik, D-37070 Göttingen, Germany and the §Max-Planck Institut für molekulare Physiologie, Postfach 102664, D-44026 Dortmund, Germany

Nucleoside-diphosphate kinases (NDKs) catalyze the transfer of γ-phosphoryl groups from NTPs via an active site histidine to NDPs using a ping-pong mechanism. We have used the change of intrinsic tryptophan fluorescence that occurs upon phosphorylation of NDK to measure the rates of phosphorylation and dephosphorylation with a range of nucleotides and nucleotide analogues. For natural nucleotides, the rates of phosphorylation and dephosphorylation were linearly dependent upon nucleotide concentration until they became too fast to measure. The second order rate constants for phosphorylation by natural NTPs varied between 0.7 and 13 x 10^6 M^-1 s^-1. Dephosphorylation by NDPs was 2–3-fold faster than the corresponding phosphorylation reaction, and dephosphorylation by dNDPs was 3–4-fold slower than the equivalent NDPs. In all cases, second order rate constants were highest for guanine followed by adenine and lowest for cytosine nucleotides. NDK also catalyzes the transfer of thiophosphate from adenineephosphate (ATP) and guanosine S'-O-(thiotriphosphate) (GTP) to NDP, but at ~1/1000 of the equivalent phosphoryl transfer rates. In this case, the observed rate constants of phosphorylation and dephosphorylation were hyperbolically dependent on nucleotide concentration. Thiophosphorylation by ATP and GTP occurred with k_max of 2.8 and 1.35 s^-1 and K_d of 145 and 36 µM respectively. For dethiophosphorylation by a range of NDPs, k_max was in the range of 5–30 s^-1, whereas K_d varied between 0.16 and 3.3 mM. Guanine had the lowest K_d values, and cytosine had the highest. The data are consistent with fast reversible binding of the nucleotide followed by the rate-limiting phosphoryl transfer. Thiophosphates change only the rate of the phosphoryl transfer step, whereas both events are influenced by the base. Modification at the S'-hydroxyl of ribose has only a small effect, while the overall rate of phosphoryl transfer is reduced 1000-fold by modification at the S'-ribose.

Nucleoside-diphosphate kinase (NDK1; EC 2.7.4.6) is a ubiquitous enzyme that catalyzes the transfer of the γ-phosphoryl group from a nucleoside triphosphate to a nucleoside diphosphate (1), involving a phosphohistidine intermediate state (2). NDK exists as either hexamers (most eukaryotic species) or tetramers (most prokaryotic species) and has been reported to display little specificity for different nucleotide bases (2, 3), reflecting a pivotal role in maintaining balanced levels of all oxy- and deoxy nucleoside triphosphates in the cell. It is thus considered a housekeeping enzyme (4), a role that has taken on greater significance with the therapeutic use of nucleotide analogues such as azidotymidine (AZT) as replication inhibitors as these compounds must be phosphorylated in the cell before becoming active (5).

In humans, two very closely (89% identity) related isoforms of NDK, designated NDK-A and NDK-B, were identified biochemically (6), which associate in vivo to form mixed hexameric isoenzymes. Evidence that NDK-A and -B may have a more complex role in the cell was provided when they were shown to be identical to the tumor suppressor proteins nm23-H1 and nm23-H2, respectively (7, 8). In addition, several point mutations in either isoform have been found in aggressive tumors (9–12), emphasizing the importance of the presence of functional NDK in the cell. Beyond their role as nucleotide kinases, a multitude of biological activities have been ascribed to these enzymes, ranging from their potential function as histidine protein kinases to the identification of NDK-B as a nucleic acid-binding protein acting as a transcription factor for the c-myc oncogene (for a review, see Refs. 13 and 14). Most recently, another member of the human nm23 tumor suppressor family, DR-nm23, has been identified; this protein seems to be involved in myeloid cell proliferation and differentiation (15). A fourth member, nm23-H4, might represent a mitochondrial isoform of human NDKs (16). Thus, the complete biological role of NDK is not clearly defined, a situation underlined by the poor understanding of the mechanism of substrate recognition and phosphate transfer by the protein.

The mechanism of the NDK activity has been extensively studied in the steady state, and a ping-pong bi-bi mechanism of NDK is well established (4, 17). Thus, the overall reaction is the sum of a donor and acceptor half-reaction, following the reaction scheme,

\[ E + \text{NDP} \rightleftharpoons E-\text{NDP} \rightleftharpoons E-P + \text{NDP} \]

where E represents NDK, E-P is phosphorylated NDK, and NTP and NDP are substrates and products and where \( K_i = \frac{k_{-i}}{k_{i}} \) is the equilibrium constant of the i-th step. However, the nature of the ping-pong mechanism with a single substrate binding site means that both of the substrates and both products are at the same level; the rates of the reactions are generally proportional to the concentration of the substrates.

This paper is available online at http://www.jbc.org
ucts compete with each other for the binding site. Thus, it is notoriously difficult to establish the true steady state parameters for such a system. This has resulted in different claims for the extent to which NDK discriminates between different substrates (2, 3, 13, 18–20). A transient kinetic approach has the potential to avoid such problems as each half-reaction can be studied in the absence of the second substrate. Under such conditions, the addition of an excess of NTP results in a single turnover of the enzyme to produce stoichiometric amounts of phosphorylated enzyme and NDP. However, few transient kinetic studies of NDK have been reported mainly because of the lack of a simple assay system for the single turnover reaction. An exception was the quenched flow study by Wälinder et al. (21) using ATP and dGDP as substrates. The recent report (22) that the phosphorylation of the active site histidine of NDK from Dictyostelium discoideum results in an enhancement of the intrinsic protein fluorescence suggested that a transient kinetic approach would now be possible.

We present here a detailed transient kinetic analysis of the human NDK-A and -B, which have been overexpressed in bacterial cells. Each half-reaction of NDK-catalyzed phosphoryl transfer was examined independently for a wide range of substrates and substrate analogues. The approach allowed the measurement of individual rate and equilibrium constants for the reaction shown above and demonstrated that for most nucleotides the enzyme binds the nucleotide rapidly and reversibly followed by rate-limiting phosphoryl transfer. In addition, the enzyme shows a significant preference for adenine and guanine nucleotides with the highest affinity for guanine nucleotides and fastest phosphoryl transfer for adenine nucleotides.

MATERIALS AND METHODS

Chemicals—Nucleotides, enzymes, and other reagents were obtained from Sigma or Boehringer Mannheim, unless stated otherwise. AZT-TP and 2’(3’)-O-(N-methyl-anthraniloyl)-ATP were generous gifts from Prof. R. S. Goody. When checked for purity by HPLC analysis (see below), ATP, ADP, GDP, and GDP were found to be more than 95% pure. ATP-S and ADP-S were more than 90% pure with the contaminating nucleotides ADP and AMP, respectively, at less than 10%.

Proteins—The cDNAs encoding NDK-A and -B (which were a generous gift from Dr. M.-L. Lacombe) were cloned into the vector pJC20 (23) without and with an N-terminal histidine tag. The pJC20 HisN vector contains the multiple cloning site of pET19b. It fuses the amino acid sequence MGH1+IDKHLD to the N terminus of the protein. This sequence includes an enterokinase cleavage site. For overproduction of recombinant proteins, these vectors were transformed into BL21 (DE3) E. coli strain. A total of 5–10 1-liter cultures were grown, and expression was induced with 1 mM isopropyl β-D-thiogalactoside for 5 h at 37 °C at an optical density of 0.5–0.7 at 600 nm. Cells were harvested by centrifugation for 15 min at 5000 rpm in a Sorvall RC-3B centrifuge and broken down by freezing and sonication. The cell debris was pelleted by centrifugation for 1 h at 11,000 rpm in an SS-34 rotor in a Sorvall RC-5B. Unmodified proteins NDK-A and -B were purified and characterized as described previously (24).

To prepare His-tagged proteins (NDK-AHis and NDK-BHis), cell extracts were dialyzed against 50 mM potassium phosphate, pH 8.0, 300 mM NaCl, containing 20 mM imidazole, and loaded onto an nickel column (Ni2+-nitrotriacetic acid-agarose; Pharmacia Biotech, Inc.). The column was washed with the same buffer containing 100 mM imidazole, and proteins were eluted with 300 mM imidazole in this buffer. The protein solutions were equilibrated in buffer A (25 mM MOPS, pH 7.2, 100 mM NaCl, 2 mM MgCl2, 1 mM EDTA, 1 mM diithiothreitol), concentrated by dialysis against solid ammonium sulfate, and stored as an ammonium sulfate precipitate at 4 °C. Proteins were recovered by gel filtration (PD10 column, Pharmacia) or by dialysis against buffer A. Purity of proteins was analyzed by standard gel electrophoresis in 15% acrylamide gels in the presence of SDS (25). Protein concentrations were estimated by measuring the absorption at 280 nm and using an extinction coefficient of 1.3 cm2 mg−1 (26) and molecular masses as given under “Results.” All protein concentrations given are with respect to catalytic sites, i.e., monomers.

Phosphorylated protein was prepared by incubating 1 ml of 20–50 μM NDK with an at least 20-fold molar excess of ATP or ATP-S for 1 min at 25 °C and then passing it through a PD10 gel filtration column (Pharmacia) which removed the nucleotides. The phosphoenzyme and thionophosphoenzyme were stable for several hours (27).

Steady-state Kinetics—NDK activity was measured at 20 °C in a standard pyruvate kinase/lactate dehydrogenase-coupled enzyme assay (4) using 2 mM ATP and 0.5 mM DTDP as substrates in buffer A. The nucleoside diphosphate substrate for NDK was DTDP because this nucleotide is a poor substrate for pyruvate kinase. An activity of 1 unit is defined as the turnover of 1 μmol of substrate in 1 min per mg of protein. The coupled enzyme assay is not suitable for following the NDK reaction with ATP and GDP as substrates, since the latter is a good substrate for pyruvate kinase. This reaction was studied by HPLC. 1 ml of experimental buffer containing 0.5–5 mM NDK-B was incubated with 5 mM ATP and 1 mM GDP. Aliquots of 40 μl were removed at fixed time intervals and the reaction was stopped by heating to 80 °C or by injecting directly into the HPLC column. Nucleotides were separated on a C-18 reversed phase column (250 × 4.6 mm, Bischoff Chromatography) filled with Hypersil 5.0 μm on a Beckman System G HPLC apparatus. The column was run at room temperature at an isotropic flow of 1.5 ml/min with 100 mM phosphate buffer, pH 6.5, 10 mM tetrabutylammonium bromide, 7.5% acetonitrile (modified after Ref. 28). Turnover rates were determined from the amount of generated GTP and ADP as a function of time.

Results—Steady-state measurements on intrinsic tryptophan fluorescence were recorded on a Perkin-Elmer LS 3B fluorimeter at 20 °C. The rates of nucleotide-induced protein fluorescence changes were measured with a Hi-Tech Scientific SF-51 or SF-61 stopped-flow rapid mixing device equipped with a 100-watt xenon/m mercury lamp and monochromator. Intrinsically tryptophan fluorescence was excited at 295 nm and detected at 90° to the incident light after passing through a WG 320 cut-off filter. A total of 512 12-bit data points were collected using a DAS 50 analogue to digital converter in a Hewlett Packard 486 computer running Hi-Tech software. 3–10 traces were collected with the same solution, averaged, and fitted to a single-exponential (Ft = F0amplitude e−kon t + F b) function using a nonlinear least-squares fitting routine. In all measurements, enzyme was mixed with at least 5-fold excess of nucleotides; if nucleotide concentrations over 1 mM were used, MgCl2 was added to ensure that Mg2+ was present in excess. Concentrations always refer to the concentrations of the reactants after mixing in the stopped-flow spectrophotometer. Experiments were performed at 20 °C in buffer A.

RESULTS

Characterization of Expressed Proteins

Gel Electrophoresis—Unmodified and N-terminally histidine-tagged NDK-A and -B were purified to greater than 90% homogeneity. The proteins (NDK-A, NDK-AHis, NDK-B, and NDK-BHis) migrated in SDS-polyacrylamide gel electrophoresis (15% SDS-polyacrylamide gel electrophoresis) as single bands at 20, 23, 19, and 22 kDa compared with calculated molecular weights (excluding the N-terminal methionine) of 17,017, 20,198, 17,167, and 20,348, respectively. Histidine-tagged proteins showed a faint second band running at the size of the untagged protein. This was probably due to cleavage of the tag at the enterokinase recognition site during the purification process; it contained less than 10% of the total protein.

Activity—When tested for activity in the coupled enzyme assay (with 2 mM ATP and 0.5 mM DTDP as substrates) at 20 °C, all proteins had specific activities of 450 units, being equivalent to turnover rates of 130 s−1. Activities of NDK-B and -BHis with 5 mM ATP and 1 mM GDP at 20 °C as determined by HPLC analysis were 120 and 136 s−1, respectively. This compares to values of 160 and 210 s−1 measured before (6) for NDK-A and -B with 1 mM ATP and 0.25 mM 8-bromo-IDP at 25 °C.

An active site titration was performed by measuring the amount of newly generated ADP with the HPLC method after incubating 14 μM NDK-BHis with 140 μM ATP. About 80% of the protein sites were phosphorylated as reported previously (17, 29).
Transient Kinetics of Nucleoside-diphosphate Kinase

Steady-state Fluorescence Studies

When 50 μM ATP was added to 1 μM NDK-B, the intrinsic tryptophan fluorescence decreased by about 10%. When phosphorylated NDK-B was prepared, the addition of 50 μM ADP resulted in a 7% increase in fluorescence. The addition of ADP to NDK-B or the addition of ATP to phosphorylated NDK-B had no measurable effect on the fluorescence, apart from a small quench due to absorption of the nucleotide. This demonstrated that the observed fluorescence changes indicated the phosphorylation state of the enzyme and not nucleotide binding (22).

In a titration experiment, the tryptophan fluorescence (excitation, 295 nm; emission, 340 nm) of 11 μM NDK-B or NDK-BHis was recorded at ATP concentrations between 0 and 300 μM (Fig. 1). The change in fluorescence (ΔF) was plotted against the added ATP concentration and used to determine K_{50%}, the concentration of ATP at which half of the maximal fluorescence change occurred, i.e. when half of the total protein is phosphorylated. A correction was made for the linear decrease in fluorescence due to absorption of the nucleotide by repeating the titration with ADP. At [ATP]_{total} = K_{50%}, half of the NDK (initially 11 μM) is phosphorylated, and therefore [NDK] = [NDK–P] = [ADP] = 11/2 μM. These values were used to calculate the overall equilibrium constant K_{eq} from the equation,

\[ K_{eq} = \frac{[NDK–P][ADP]}{[NDK][ATP]} \]  

(Eq. 1)

where [ATP] = [ATP]_{total} = 5.5 μM. The values of K_{50%} were 14.9 ± 1 μM for NDK-B and 15.7 ± 1 μM for NDK-BHis. Thus, K_{eq} = 0.59 ± 0.05 was obtained for NDK-B, and K_{eq} = 0.54 ± 0.05 was obtained for NDK-BHis.

NDK-A showed a similar quenching in fluorescence upon adding ATP; however, the change in fluorescence was smaller (~7% upon addition of ATP) than for NDK-B. Due to this small signal, K_{eq} was not determined for NDK-A.

Transient Kinetic Studies

Phosphoryl Transfer Reactions of NDK-A and -B with ADP and ATP—Since the addition of ATP leads to a 10% decrease in protein fluorescence, this signal can be used to follow the rate of the ATP-induced phosphorylation reaction. Fig. 2a shows the changes in fluorescence recorded upon mixing 0.5 μM NDK-BHis with 10 μM ATP in the stopped-flow fluorimeter. A 9% decrease in fluorescence was observed, which was well described by a single exponential function with an observed rate constant (k_{obs}) of 88 s⁻¹. When ADP replaced ATP, no significant change in fluorescence occurred. In an analogous experiment, 0.5 μM phosphorylated NDK-BHis (NDK-BHis–P) was mixed with 5 μM ADP and led to a 6% increase in fluorescence, with k_{obs} = 119 s⁻¹ (Fig. 2b). The addition of ATP to phosphorylated enzyme produced no change in fluorescence.

The measurement was repeated for nucleotide concentrations between 2.5 and 30 μM for ATP and 2 and 10 μM for ADP. At even higher concentrations, the reaction was difficult to measure, since the amplitude of the reaction gradually disappeared, limited by the 2-ms dead time of the stopped-flow system. Over these concentration ranges, k_{obs} was linearly dependent on nucleotide concentration (Fig. 3, a and b). The linear dependence of k_{obs} suggests that the change in fluorescence was limited by the rate of nucleotide binding, and the slope of the best fit line defines the apparent second order rate constant for the nucleotide binding reaction. A provisional assignment of this second order rate constant in terms of the intrinsic rate constants of Scheme 1 can be made with a few assumptions: 1) the fluorescent signal originates from the formation of the phosphorylated protein; and 2) the reverse reaction is negligible because the concentration of ADP is very low throughout the transient reaction (reaching a maximum of 0.5 μM, the concentration of the protein). In this case, there are two situations in which a single exponential is expected for the fluorescence transient and in which k_{obs} is linearly dependent on ATP concentration.

1) When the phosphor transfer reaction is much faster than nucleotide binding (i.e. k_{1,2} + k_{2,1} ≫ [ATP] k_{k,1} + k_{k,1}), then the apparent second order rate constant is given by k_{k,1}. This predicts that the event observed is a diffusion-controlled reaction, and as such k_{k,1} should have a value of 10^7 to 10^8 M⁻¹s⁻¹. Instead, the reaction should have little dependence on temperature (30). In fact, the second order rate constant is of the order expected for a diffusion-controlled reaction for ADP and GDP, as shown below, but this is not true of all other nucleotides (see below).

2) When phosphor transfer is much slower than nucleotide binding (i.e. k_{k,1} + k_{k,1} ≫ [ATP] k_{k,1} + k_{k,1}), then k_{obs} is predicted to be hyperbolically dependent on [ATP],

\[ k_{obs} = k_{k,1}[ATP] / (1 + K_{1}[ATP]) \]  

(Eq. 2)

and at low ATP concentrations, such that K_{1} \times [ATP] ≪ 1, then k_{obs} = k_{k,1}[ATP], and the apparent second order rate constant defines K_{1,2,1}.

The model in which k_{k,1} + k_{k,1} ≪ [ATP] k_{k,1} + k_{k,1} can be eliminated, since this would produce significant deviations from a single exponential change in fluorescence. The data for ATP and ADP (Fig. 3) do not clearly distinguish cases 1 and 2. Preliminary data at 10 °C suggest the reaction slows down by a factor of 2, which again does not disting that the two possible interpretations. However, in general we favor situation 2, because the values of the second order rate constants observed for a wide range of nucleotides, discussed below, are much too slow for a diffusion-controlled reaction.

The same measurements were repeated for untagged NDK-B and untagged and His-tagged NDK-A, and the data are summarized in Table I. All proteins have similar rates of phosphorylation; however, dephosphorylation was 2–3-fold faster for NDK-B compared with NDK-BHis, and for untagged and His-tagged NDK-A. The amplitudes of the fluorescence changes were not altered by the His-tag, and NDK-A had an approximately 2-fold smaller amplitude than NDK-B. Since the differences between the tagged and untagged proteins are small, the remainder of the measurements presented here have used the more easily produced NDK-BHis.

The results presented in Table I show that for the ATP/ADP...
substrate pair, the dephosphorylation reaction is 2–5 times faster than the phosphorylation reaction, leading to \( K_{\text{eq}} \) (equal to \( k_{\text{phos}}/k_{\text{dephos}} \)) = 0.2–0.67 for all constructs, which is in broad agreement with the titration data (Fig. 2). These values are in the same range as determined for D. discoideum NDK of 0.2 (22), for yeast NDK of 0.19 (17), and for pig heart NDK of 0.25 (31).

Phosphoryl Transfer Reactions of NDK-B with Natural Substrates—The reactions shown in Fig. 2, a and b, were repeated for a wide range of naturally occurring nucleotides and in each case, the reaction could be described by a single exponential. The plots of \( k_{\text{obs}} \) against the nucleotide concentration are shown in Fig. 3. The apparent second order rate constants obtained from the slopes of the plots are summarized in Table II. For the nucleotides with lower values of \( k_{\text{obs}} \) (C, U, and T nucleotides), the concentration range was extended well beyond that shown in these plots. In all cases, the plots were linear over the accessible range. It is important to note that for most of the nucleotides used the values of the intercepts from these plots are not significantly different from 0. Only ATP, GTP, dADP, and dGDP have significant intercepts, and this suggests that the assumption of a negligible reverse reaction is not completely valid in these cases. For the model of Scheme 1, in which both nucleotide binding events are much faster than the phosphoryl transfer step, the complete solution to the rate equations can be given for the conditions where both \( K_i \) [NTP] and [NDP]/\( K_i \) are \( \ll 1 \) and the reaction is pseudo-first order in both directions. Thus the following is true.

\[
k_{\text{obs}} = K_i k_{\text{phos}} [\text{NTP}] + k_{\text{dephos}} \text{[NDP]} / K_i \quad \text{(Eq. 3)}
\]

When NTP is varied, the slope of a plot of \( k_{\text{obs}} \) versus [NTP] defines \( K_{\text{phos}} \) and the intercept \( k_{\text{dephos}} [\text{NDP}] / K_i \). Of course, the reactions shown in Fig. 3 are not first order in the reverse direction, but the above equations allow an estimate of the maximum value of the intercept. If the value of \( k_{\text{dephos}} K_i \) is taken from the slope of the data with NTP, it can be used to predict the value of the intercept of the NTP plot by assuming that the \([\text{NDP}] = [E]_0 = 0.5 \mu M\). The values are thus 8.9 and 14.5 s\(^{-1}\) for ATP and GTP, respectively, and they are in good agreement with the corresponding intercept values. All other nucleotides predict much lower intercepts, which would not be significantly above 0 in the plots of Fig. 3.

The results in Table II demonstrate that NDK-B has clear preferences for certain nucleotides, with the apparent second order rate constants differing by more than 100-fold. This is not consistent with the events being diffusion-controlled, since diffusion is similar for all nucleotides. Within one set of nucleotides (phosphorylation with NTP, dephosphorylation with NDP or dNDP), the initial rates were always fastest for guanine substrates and were followed by adenine, uracil, and cytosine with relative rates of 1.0, 0.55, 0.12, and 0.04 respectively. In addition, rates of dephosphorylation were about 3-fold faster for NDP than for dNDP. Rates for dephosphorylation were about 2-fold faster than phosphorylation, such that for each substrate/product pair the overall equilibrium constant was 0.5 ± 0.12.

Many of the experiments with the different nucleotides were repeated with both untagged and tagged NDK-A and NDK-B. None of the observed second order rate constants differed by more than a factor of 2 from those reported for NDK-B\(_{\text{His}}\).

Phosphorylation of NDK-B with Ribose-modified Nucleotides—To clarify the ability of NDK-B to recognize different substrates, we examined the phosphorylation reaction for a series of NTPs modified on the ribose ring. We compared ATP, 2'-deoxy-ATP (dATP), and 3'-deoxy-ATP (Cordycepin). All nu-
cleotides phosphorylated the protein as observed by the increase in protein fluorescence, and $k_{\text{obs}}$ was linearly dependent upon nucleotide concentration. The apparent second order rate constants were $7.9 \times 10^6$ and $2 \times 10^6$ s$^{-1}$ M$^{-1}$, respectively. In contrast, the addition of 1 mM 2',3'-dideoxy ATP gave no increase in protein fluorescence, indicating that it may not be a substrate for NDK. The reaction of 3'-azido-3'-deoxythymidine triphosphate (a substrate for human immunodeficiency virus reverse transcriptase, leading to chain termination) with NDK gave an apparent second order rate constant of $7 \times 10^4$ s$^{-1}$ M$^{-1}$, which is $\frac{1}{3}$ of the rate with 3'-dATP, showing that the bulky azide group can be accommodated comparably well into the binding cleft. A similar result was observed for methyl anthraniloyl ATP (mixed 2'- and 3'-isomers), which phosphorylated NDK at $\frac{1}{3}$ of the rate of 2'-dATP. These results are in broad agreement with those of Bourdais et al. (32) using a steady-state approach.

**Phosphoryl Transfer Reaction with Thiomodified Nucleotide Analogues—Thiomodified nucleotides (ATP$\gamma$S and GTP$\gamma$S) are substrates for NDK (33), and indeed the thiophosphorylated enzyme can be isolated and is as stable as the natural complex. This modification (substitution of sulfur for oxygen) has been shown to reduce the rate of phosphoryl transfer for a wide variety of enzymes (34).

As seen for natural nucleotides, the fluorescence of NDK-BHis decreased on mixing with excess ATP$\gamma$S and increased again when the thiophosphorylated enzyme was mixed with NDP. However, the amplitude of the fluorescence change was smaller than that observed with normal NTP. In stopped-flow experiments, fluorescence changes could be well described by a single exponential, but the values of $k_{\text{obs}}$ were generally 500-fold slower than those of the equivalent reaction with NTP or NDP (Fig. 2, c and d). For example, the addition of 50 $\mu$M ATP$\gamma$S to 0.5 $\mu$M NDK-BHis resulted in a $7\%$ decrease in fluorescence with $k_{\text{obs}} = 0.7 \text{ s}^{-1}$. Similarly, the addition of 50 $\mu$M ADP to 0.5 $\mu$M thiophosphorylated NDK-BHis (NDK-BHis-thioP) produced a 4% increase in fluorescence with $k_{\text{obs}} = 1.8 \text{ s}^{-1}$. However, in contrast to the reactions with normal nucleotides, the dependence of $k_{\text{obs}}$ on nucleotide concentration was no longer linear but saturated at high nucleotide concentrations for both NTP and NDP (Fig. 4, a and b). The dependence could be described by a hyperbolic function,

$$k_{\text{obs}} = \frac{[\text{NXP}]k_{\text{max}}(K_{0.5} + [\text{NXP}])}{1 + (K_{0.5} + [\text{NXP}])}$$

(Eq. 4)

where NXP is NDP or NTP, $k_{\text{max}}$ is the maximum value of $k_{\text{obs}}$, and $K_{0.5}$ is the concentration of nucleotide required for $k_{\text{obs}} = \frac{k_{\text{max}}}{2}$.

This form of the concentration dependence of $k_{\text{obs}}$ is that expected for a two-step reaction as described in Equation 2. Since the presence of the thiophosphate group is likely to slow down the phosphoryl transfer ($k_{-2}$ and $k_{2}$), it is reasonable to assume that it is this step that is limiting here. In such a case, then $K_{0.5} = 1/K_1$ and $k_{\text{max}} = k_{-2}$ for NTP$\gamma$S (Scheme 1), and $K_{0.5} = K_5$ and $k_{\text{max}} = k_{-2}$ for thiophosphoryl transfer to NDP. The values of $K_{0.5}$ (defining the dissociation constant, $K_d$), $k_{\text{max}}$, and the apparent second order rate constant $k_{\text{obs}}/K_{0.5}$ are summarized in Table III.
Phosphorylation of thiophosphorylated NDK-B His

rkylation of 0.5

tions above 1 mM were used, MgCl₂ was added to the buffer (see Fig. 1)
maintained for the thiophosphoryl reaction;
tween the second order rate constants for the four NDPs is
seen for the natural nucleotides. Similarly, the relationship be-

against substrate concentrations between 10 and 3000
msg 2

to ensure that Mg 2

modified substrates.
Observed rate constants (kₒ), superim-
posed on the hyperbolic curve, which is shown superim-
posed, to obtain Kₒ,5 and kₘₐₓ (Table III). Where nucleotide concentra-
tion was present in excess over the nucleotide.

The data in Fig. 4 and Table III show that the values of the
apparent second order rate constants for the NTPS, yielding Kₒ,5
values of 469 ± 72 μM and 168 ± 28 μM and kₘₐₓ of 106 ± 11
s⁻¹ and 34 ± 2 s⁻¹, respectively. No transfer of a thiophospho-
ryl group from NDK-Bₜₛₜ-P to ADPβS was detected by
HPLC analysis of a mixture of 5 mM ATPβS, 1 mM ADPβS, 1 μM
NDP-Bₜₛₜ after 0.5- and 2-h incubation. 1 unit of alkaline
phosphatase was added to remove nonthiophosphate nucleo-
tides present. The values of Kₒ,5 for thiophosphorylation of GDP
and phosphorylation of GDPβS are very similar, yet ADPβS
appears to bind more tightly to the phosphoenzyme than ADP
binds to the thiophosphorylated enzyme. The phosphoryl trans-
fer step (kₒ,2) was much slower than dephosphorylation (kₒ,1) and Kₒ,2 (equal to kₒ,1/kₒ,2) has a value of 0.08 and 0.17 for
ATPβS and GTPβS, respectively. The NTPβS bound approxi-
ately 5-fold more tightly (Kₒ,1) than the equivalent dipho-
phate (1/Kₒ,2), bringing the Kₒ,5 values back to 0.6 and 0.76 for
ATPβS and GTPβS. The affinity of guanine nucleotides was
highest, and that of adenine was 4–6-fold weaker. The 1/Kₒ,5
values for UDP and CDP were not firmly established, because
complete saturation could not be reached; they were 15–20-fold
weaker than GDP.

When dNDP was used to dephosphorylate NDK-Bₜₛₜ-thioP,
the values of Kₒ,5 did not change significantly from those for
NDP apart from dUDP, which bound about 4-fold more tightly.
However, the maximal rate, kₘₐₓ was reduced about 7-fold,
and the apparent second order rate constant was reduced about
6-fold compared with NDP (Table III and Fig. 4, b and c).
Notably, dTDP and dADP gave very similar results when follow-

phosphorylation of NDK-Bₜₛₜ-thioP (Fig. 4c).
The thiomodified nucleoside diphosphates, ADPβS and
GDPβS, were also examined and could be phosphorylated by
NDK-Bₜₛₜ-P to form the products ATPβS and GTPβS. The kₒ,5
was again hyperbolically dependent on [NDPβS], yielding Kₒ,5

ATPβS GTPβS ADP GDP UDP CDP
19 38 22 49 6 1.7
35 36 42 55 83 15
4.8 ± 0.07 1.35 ± 0.04 33.5 ± 1.7 8.1 ± 0.1 12.9 ± 0.8 5.5 ± 1

TABLE III
Kinetic parameters for thiophosphoryl substrates and products

| Substrate   | kₒ,5 (μM) | kₒ,5 (s⁻¹) |
|-------------|-----------|------------|
| ATPβS       | 145 ± 15  | 2.81 ± 0.07 |
| GTPβS       | 36 ± 4   | 1.35 ± 0.04 |
| ADP         | 1050 ± 650 | 33.5 ± 1.7  |
| GDP         | 165 ± 5  | 8.1 ± 0.1  |
| UDP         | 2320 ± 325 | 12.9 ± 0.8  |
| CDP         | 3249 ± 815 | 5.5 ± 1    |
| dADP        | 1300 ± 400 | 4.7 ± 0.43  |
| dGDP        | 192 ± 28  | 1.4 ± 0.1  |
| dUDP        | 612 ± 133 | 0.96 ± 0.09 |
| dCDP        | 2130 ± 1372 | 0.7 ± 0.2  |
| dTDP        | 900 ± 590 | 6.0 ± 0.4  |
| ADPβS       | 469 ± 72  | 106 ± 11   |
| GDPβS       | 168 ± 28  | 34 ± 2     |

The apparent second order rate constant is separated into its
two components, a different pattern emerges. The phospho-
ryl transfer step is 2–5 times faster for adenine nucleotides
than for the other three examined, which were all similar to
within a factor of 2. For adenine and guanine, the thiophospho-

Fig. 4. Secondary plot of kₒ,5 against concentrations of thi-
modified substrates. Observed rate constants (kₒ,5) are plotted
against substrate concentrations between 10 and 3000 μM for phospho-
ylation of 0.5 μM NDK-Bₜₛₜ with ATPβS and GTPβS (a) and for de-
phosphorylation of thiophosphorylated NDK-Bₜₛₜ-thioP with NDP (b)
and dNDP (c). Nucleotides had the base moieties guanine (●), adenine
( ◦), uracil (▲), thymine (▼), and cytosine (■). Conditions were as in Fig.
2. Data were fitted with a hyperbolic curve, which is shown superim-
posed, to obtain Kₒ,5 and kₘₐₓ (Table III). Where nucleotide concentra-
tions above 1 mM were used, MgCl₂ was added to the buffer (see Fig. 1)
to ensure that Mg²⁺ was present in excess over the nucleotide.

The thiomodified nucleoside diphosphates, ADPβS and GDPβS, were also examined and could be phosphorylated by
NDK-Bₜₛₜ-P to form the products ATPβS and GTPβS. The kₒ,5
was again hyperbolically dependent on [NDPβS], yielding Kₒ,5
values of 469 ± 72 μM and 168 ± 28 μM and kₘₐₓ of 106 ± 11
s⁻¹ and 34 ± 2 s⁻¹, respectively. No transfer of a thiophospho-
ryl group from NDK-Bₜₛₜ-thioP to ADPβS was detected by
HPLC analysis of a mixture of 5 mM ATPβS, 1 mM ADPβS, 1 μM
NDP-Bₜₛₜ after 0.5- and 2-h incubation. 1 unit of alkaline
phosphatase was added to remove nonthiophosphate nucleo-
tides present. The values of Kₒ,5 for thiophosphorylation of GDP
and phosphorylation of GDPβS are very similar, yet ADPβS
appears to bind more tightly to the phosphoenzyme than ADP
binds to the thiophosphorylated enzyme. The phosphoryl trans-
fer step was about 3-fold faster for phosphorylation of the two
NDPβS than was thiophosphorylation of the corresponding NDP.

Phosphorylation with ATPβS and dephosphorylation of thiophosphorylated NDK with ADP and GDP were determined for
unlagged NDK-B, showing that kₘₐₓ and Kₒ,5 were within 10% of those for NDK-Bₜₛₜ. NDK-A was phosphorylated with
ATPβS and GTPβS, and values obtained for Kₒ,5 were 250 ± 100 and 92 ± 8 μM, and values for kₘₐₓ were 3 ± 0.4 and
1.95 ± 0.1 s⁻¹, respectively. Values for Kₒ,5 of NDP-A com-
pared with NDP-B were 2–3-fold higher, but the maximal rates
were similar. Also, for NDP-A the Kₒ,5 of GTPβS was about
2.5-fold lower than that of ATPβS; in the case of NDP-B,
GTPβS was bound 4-fold more tightly than ATPβS. The amplitudes
for the fluorescence changes of NDP-A were too small to get reliable values for dephosphorylation of thiophosphorylated
NDK-A with NDP.
DISCUSSION

Human NDK-A and -B have been expressed and purified from Escherichia coli as both unmodified and His-tagged proteins. All four constructs behave in a similar way, suggesting that the His tag has no major influence on the activity of the proteins in the steady state. Since the NDK-B1his was the easiest to purify, the detailed kinetic analyses were all performed on this construct.

Phosphoryl Transfer Measured by the Intrinsic Fluorescence Change—We have shown that, as for the D. discoideum protein (22), phosphorylation of the active site histidine in the human enzyme (Hia115) results in a quenching of the intrinsic protein fluorescence, which is reversed on dephosphorylation. Human NDK-A and -B contain three tryptophan residues at positions 78, 133, and 149 (35). The D. discoideum NDK has only a single tryptophan at position 137 (equivalent to position 133 in human NDK) and shows a larger (20%) quench of fluorescence on phosphorylation (22). This suggests that the 10% fluorescence quench of the human enzyme originates primarily from the Trp133. Since this Trp is conserved in almost all eukaryotic species, a similar fluorescence change may be expected for other NDKs, so the transient kinetic approach developed here may be widely applicable.

We used this fluorescence change to study the mechanism of the enzyme with transient kinetic methods. Unlike steady-state methods, this approach can study each half-reaction independent of the competing back reaction. This is a major advantage for an enzyme with a ping-pong mechanism. All of the kinetic data shown here are consistent with reaction Scheme 1.

The fluorescent transients observed for all four NDK constructs in the stopped-flow studies were single exponentials, and $k_{obs}$ was linearly dependent on nucleotide concentration over the accessible range (between 2 and 10 $\mu$M for GTP). Thus, the observed event is limited by the access of the nucleotide to the protein. However, except for ADP and GDP, each of the second order rate constants was significantly lower than that expected for a diffusion-controlled reaction. This point is emphasized by the results with other natural (Table II) and non-natural (Table III) nucleotides in which the constant varied by a factor of 250–500 s$^{-1}$ for ATP, GTP, and GDP, respectively. Similar calculations can be made for all of the other nucleotides in Tables II and III and suggest that for UDP, CDP, DADP, and dTDP, $k_{obs}$ is similar to that of GDP (3000–6000 s$^{-1}$) and is 500–1000 s$^{-1}$ for dGDP, dUDP, and dCDP. Thus, the results for the wide range of nucleotides (GTP, ATP, GDP, and dGDP) and >1 mM (other nucleotides) are consistent with the prediction from the GTP$^1$S data.

The above discussion compares the relative rates of the nucleotide binding/dissociation step, which occurs before phosphoryl transfer obtained from studies of each half-reaction in transient kinetic experiments, i.e. when the product dissociation does not influence the observed events. To extrapolate to steady-state conditions, the product dissociation reaction must be considered as well as the major problem with all steady-state studies of ping-pong mechanisms, substrate inhibition. In our case, this is the binding of NTP to NDK and NTP to phosphorylated NDK. This work provides no estimates of the inhibition constant values for the substrates, but the approach used here could provide estimates by examining the competitive inhibition of ATP$^1$S when dephosphorylating phosphorylated NDK with NTP or of ADP$^1$S when phosphorylating NDK with NTP. The data presented here do allow us to state that for all deoxynucleotides and for pyrimidine nucleotides, phosphoryl transfer will be rate-limiting. For those nucleotides, the enzyme has rapid equilibrium binding of substrates and $k_{cat} = k_{cat} \cdot k_{dissoc}(k_{cat} + k_{dissoc})$. In the case of adenine and guanine, the nucleotide dissociation steps may be slow enough to influence the steady-state rates.

Influence of Base and Sugar Moieties on Nucleotide Binding and Phosphoryl Transfer—The results for the wide range of nucleotides (Fig. 3, Tables II and III) show clear differences in the way the base is recognized by NDK-B. The apparent second
order rate constants were always highest with guanine nucleotides, followed by adenine and uracil nucleotides, and lowest with cytosine nucleotides with the relative rates of 1, 0.55, 0.12, and 0.04. Furthermore, dephosphorylation was always twice as fast as phosphorylation, with the same nucleotide giving the expected invariant $K_m$ Thus, although the free energy of phosphoryl transfer is the same in each case, the enzyme clearly distinguishes the different bases. The discrimination between the bases by NDK has not been well established before, because the problem with competitive inhibition between substrates mentioned in the introduction and the coupled enzyme assay, which is predominantly used to measure steady-state activity, allows only certain pairs of substrates to be used. Thus, it was only claimed that NDK is nearly completely unspecific (3, 36, 37), despite some studies showing highly varying values of $K_m$ with different nucleotides for human NDK (18).

Studies with triphosphoryl nucleotides reveal that the effects of the base on the second order rate constants contain a contribution from both a change in the affinity of the nucleotide and a change in the phosphoryl transfer step. Thus, it was observed that at low nucleotide concentrations, ATP and GTP have the highest $k_{\text{max}}$ but ADP and GDP have the highest $k_{\text{max}}$ (e.g. 20-fold compared with 6-fold for NTPs). Similarly the loss of the 2'-hydroxyl on the ribose causes an 8-fold reduction in $k_{\text{max}}$ and in each case a small increase in $K_m$ for dADP, dGDP, and dCDP but a significant decrease for dUTP.

**Structure-Function Relationships**—The interactions of the base and the sugar of the nucleotide with NDK have been revealed in crystal structures of NDK with bound dTDP (NDK of *D. discoideum* (37)), ADP (NDK of *D. discoideum* (36); NDK of *Myxococcus xanthus* (38)), and GDP (NDK-B (39)). The base of the nucleotide is wedged between Phe 60 and Val 112 and of revealed in crystal structures of NDK with bound dTDP (NDK and dCDP but a significant decrease for dUDP. 

Glu152 (numbering refers to human NDK) can form a hydrogen bond directly with GDP or via a water molecule with ADP and Steeg, P. S. (1991) *Cancer Res. 51*, 445–449. 

Leone, A., Seeger, R. C., Harrison, M. H., Ho, Y. Y., Arbeloja, M. J., Brodeur, G. M., Stram, D. J., and Steeg, P. S. (1993) *Oncogene* 8, 855–865.

Chang, C. L., Zhu, X. Y., Thorval, D. H., Ungar, D., Rawwas, J., Hora, N., Strahlur, J. R., Hanash, S. M. (1994) *Nature* 370, 335–336.

Hambý, C. V., Mendola, C. E., Potla, L., Stafford, G., and Backer, J. M. (1995) *Biochem. Biophys. Res. Commun.* 211, 579–586.

Mandell, M., Konishi, I., Komatsu, T., Mott, T., Aron, S., Nomura, H., Kanda, Y., Hiai, H., and Fukumoto, M. (1995) *Brit. J. Cancer 72*, 691–695.

Liotta, L. A., and Steeg, P. S. (1990) *J. Natl. Cancer Inst. 82*, 1170–1172.

De La Rosa, A., Williams, R. L., and Steeg, P. S. (1995) *BioEssays* 17, 53–62.

Venturelli, D., Martinez, R., Melotti, P., Casella, I., Peschle, C., Cucco, C., Spampinato, G., Darzyynkiewicz, Z., and Calabretta, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7425–7429.

Milo, I., Rousseau-Merx, M. F., Monier, A., Ment, L., Lacombe, D., Caseau, J., and Lacomb, M.-L. (1997) *Human Genet.* 99, 550–557.

Garcés, E., and Céland, W. W. (1969) *Biochim. Biophys. Acta* 109, 2139–2143.

Bominar, A. A., Molién, A. C., Pestel, M., Veron, M., and Van Haastert, P. J. (1993) *EMBO J.* 12, 2275–2279.

Kowluru, A., and Metz, S. A. (1994) *Biochem. Biophys. Res. Commun.* 202, 1249–12503.

Wölnder, O., Zetterqvist, O., and Engström, L. (1969) *J. Biol. Chem.* 244, 1090–1094.

Deville-Bonne, D., Sellam, O., Merola, F., Lascu, I., Desmadrill, M., and Veron, M. (1996) *Biochemistry* 35, 14634–14650.

Clos, J., Westwood, J. T., Becker, P. G., Wilson, S., Lambert, K., and Wu, C. (1990) *Cell* 63, 1085–1097.

Schäfer, S. (1996) *FEMS Lett.* 394, 316–320.

Laemmli, U. K. (1970) *Nature* 227, 680–685.

Gill, S. C., and von Heijl, P. H. (1989) *Anal. Biochem.* 182, 319–326.

Morera, S., Schiattani, I. Vetter, B. R., Lascu, I., and Janin, J. (1995) *Biochemistry* 34, 11062–11070.

Tucker, J., Szakéki, G., Feuerstein, J., John, J., Goody, R. S., and Wittinghofer, A. (1996) *EMBO J.* 15, 3559–3569.

Lecomte, H., Lascu, I., Bominar, A. A., Veron, M., and Delpechier, M. (1995) *Biochemistry* 34, 12445–12450.

Gutfreund, H. (1995) *Kinetics in the Life Science*, pp. 261–275, Cambridge University Press, Cambridge, UK.

Lascu, I., Pop, R. D., Porup, H., Prescne, E., and Proinov, I. (1983) *Eur. J. Biochem.* 135, 487–503.

Bourdais, J., Biondi, R., Sarfatti, S., Guerrier, C., Lascu, I., Janin, J., and Veron, M. (1996) *J. Biol. Chem.* 271, 7878–7890.

Goody, R. S., Eckstein, F., and Schirmer, B. H. (1972) *Biochim. Biophys. Acta* 270, 155–161.

Frey, P. A. (1990) *Adv. Enzymol.* 62, 119–199.

Hama, H., Almawi, N., Lerner, C. G., Inouye, S., and Inouye, M. (1991) *Gene* 105, 31–36.

Morera, S., Lascu, I., Dumas, C., Le Bras, G., Brozio, P., Veron, M., and Janin, J. (1994) *Biochemistry* 33, 459–467.

Cherfils, J., Morera, S., Lascu, I., Veron, M., and Janin, J. (1994) *Biochemistry* 33, 9062–9069.

Williams, R. L., Oren, D. A., Mounoz-Dorado, J., Inouye, S., and Arnold, E. (1993) *J. Mol. Biol.* 234, 1230–1247.

Morera, S., Lascu, I., Yungwu, X., Le Bras, G., and Janin, J. (1995) *Structure* 3, 1307–1314.

Lavie, A., Vetter, I. R., Konrad, M., Goody, R. S., Steinheit, J., and Schlichting, I. (1997) *Nat. Struct. Biol.* 4, 601–604.

Lavie, A., Schlichting, I., Vetter, B. R., Konrad, M., Reinstein, J., and Goody, R. S. (1997) *Nat. Med.* 3, 922–924.

Wu, Y. W., Sellam, O., Morera, S., Sarfatti, S., Biondi, R., Veron, M., and Janin, J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 7162–7165.