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Human Nucleoside Diphosphate Kinase B (Nm23-H2) from Melanoma Cells Shows Altered Phosphoryl Transfer Activity Due to the S122P Mutation*

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The Ser^{122} → Pro mutation in human nucleoside diphosphate kinase (NDK)-B/Nm23-H2 was recently found in melanoma cells. In comparison to the wild-type enzyme, steady state activity of NDK^{S122P} with ATP and TDP as substrates was slowed down 5-fold. We have utilized transient kinetic techniques to analyze phosphoryl transfer between the mutant enzyme and various pairs of nucleoside triphosphates and nucleoside diphosphates. The two half-reactions of phosphorylation and dephosphorylation of the active site histidine residue (His^{118}) were studied separately by making use of the intrinsic fluorescence changes which occur during these reactions. All apparent second order rate constants are drastically reduced, falling 5-fold for phosphorylation and 40–200-fold for dephosphorylation. Also, the reactivity of the mutant with pyrimidine nucleotides and deoxy nucleotides is more than 100-fold reduced compared with the wild-type. Thus, the rate-limiting step of the NDK-B^{S122P}-catalyzed reaction is phosphoryl transfer from the phospho-enzyme intermediate to the nucleoside diphosphate and not phosphoryl transfer from the nucleoside triphosphate to the enzyme as was found for the wild-type protein. This results in a pronounced shift of the equilibrium between unphosphorylated and phosphorylated enzyme. Moreover, like the *Killer-of-prune* mutation in *Drosophila* NDK and the neuroblastoma Ser^{120} → Gly mutation in human NDK-A/Nm23-H1, the Ser^{122} → Pro substitution in NDK-B affects the stability of the protein toward heat and urea. These significantly altered properties may be relevant to the role of the mutant enzyme in various intracellular processes.

Nucleoside diphosphate kinases (NDK)[1] catalyze the transfer of the γ-phosphoryl group from nucleoside triphosphates to nucleoside diphosphates via an enzyme intermediate state with the phosphoryl group bound covalently to a histidine residue in the active site. The enzymatic properties of NDKs have been examined in many early studies (1); however, recently a completely new role for this enzyme has emerged when the product of the tumor suppressor gene *nm23* was identified as NDK (2, 3). Since then, several point mutations in NDK have been found in tumor cell lines: S120G in the human isoform NDK-A (4), L48V (5), S122P (6), and W133R (7) in the human isoform NDK-B. So far, only NDK-A^{S120G}, which was found in neuroblastoma, has been characterized extensively in two independent studies (8, 9), demonstrating that its enzymatic properties are similar to the wild-type protein. In contrast, its stability is significantly reduced as observed under various *in vitro* conditions (9). However, these studies could not explain satisfactorily the highly metastatic behavior of the tumor cells expressing mutated NDK-A^{S120G}. The NDK-B^{S122P} mutant was identified in melanoma of high metastatic potential as tested by transfection into mice (6). It was speculated that this mutant protein has a quarternary structure different from the wild-type, but so far no data were available about effects of this mutation on enzymatic activity or stability. Another point mutation known in *Drosophila* NDK, P97S, called *Killer of prune* (*Kpn*) mutation, has been shown to affect severely the development of the fly (10). This mutation reduces stability but not catalytic efficiency of the protein (11).

The mechanism of tumor suppression by NDK is poorly understood, although some speculations about the role of the enzyme have been presented (12, 13). The effects of mutations leading to metastatic tumor cell lines are not clear, even though there is evidence from *Drosophila* NDK mutants that stability of the oligomeric enzyme may play a major role (11). In the present study we applied transient kinetic methods to examine the phosphoryl transfer reactions of the mutants NDK-B^{S122P} and NDK-A^{S120G}, an approach we successfully used before to characterize human wild-type NDKs (14). We measured the phosphorylation and the dephosphorylation reactions of both mutant enzymes with different substrates and compared them with the wild-type enzymes under identical experimental conditions. Whereas NDK-A^{S120G} had only slightly altered enzymatic properties, NDK-B^{S122P} showed major changes in the reaction mechanism. This, together with a reduced stability toward heat and urea, may shed light on this mutant protein and the associated metastatic potential.

MATERIALS AND METHODS

Mutagenesis and Cloning—All reagents were obtained from Sigma and Roche Molecular Biochemicals, unless stated otherwise, and treated as described before (14). The mutations S122P and S120G were introduced by site-directed mutagenesis after the Kunkel Method (15) using the Muta-Gene kit (Bio-Rad) and the mutagenic primers: 5′-CATGGCAGTGAT***CCG***GTAAAAAGTGCTG-3′ for S122P and 5′-GAA-

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[1] The abbreviations used are: NDK, nucleoside diphosphate kinase; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; NTPγS, nucleoside 5′-*O*-(3-thiotriphosphate); WT, wild type; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); MOPS, 4-morpholinepropanesulfonic acid.

CATTATACATGGC***G***GTGATTCTGT-3′ for S120G (9). The altered bases are indicated in bold italic letters, and the underlined sequences specify the newly introduced restriction sites for *Bsa*WI and *Hph*I, respectively, which were used to identify positive clones carrying the mutation. The entire coding regions of the mutants were verified by automatic DNA sequencing (Applied Biosystems 373 sequencer) to confirm the presence of the desired mutation and clone integrity. The DNA fragments encoding WT and mutant NDK-A were cloned into the *Escherichia coli* expression vector pJC20 (16, 17) and that for WT and mutant NDK-B into pJC20 HisN. This latter vector was generated by transferring the 125 bp *Xba*I/*Bam*HI fragment (which encompasses the ribosomal binding site, the histidine tag region, and the enterokinase cleavage site) from the pET-19b vector (Novagen) into the pJC20 vector cut with *Xba*I and *Bam*HI. Cloning of an open reading frame via *Nde*I and *Bam*H restriction sites into pJC20HisN then fuses the amino acid sequence $MG(H)_{10}I(D)_4KHLD$ to the N terminus of the protein.

Protein Preparation and Analysis—Proteins were overproduced in BL21 (DE3) *E. coli* strain after induction with 1 mM isopropyl-β-D-thiogalactopyranoside. The NDK-B proteins carrying the N-terminal histidine-tag (His-tag) were purified on a nickel-agarose column (Qiagen) according to the instructions of the supplier. The 21-amino acid N-terminal extension was shown to have no influence on the activity of the enzyme (14). Mutant NDK-A was purified like wild-type NDK-A as described before (9). Proteins were equilibrated in buffer A (buffer A: 25 mM Tris-Cl, pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) and stored as an ammonium sulfate precipitate at 4 °C. Phosphorylated NDK was prepared by incubating the protein with an excess of nucleoside triphosphates and then passing it through a PD10 gel filtration column (Amersham Pharmacia Biotech) that removed the nucleotides. The phosphorylated proteins was stable for several hours when kept on ice. Protein concentrations were estimated by using an extinction coefficient of 1.3 cm^2 mg^{-1} at 280 nm (18) and molecular masses of 17.1 kDa for NDK-A and 17.2 kDa for NDK-B and are given with respect to catalytic sites, *i.e.* monomers. Proteins were analyzed by 15% SDS-polyacrylamide gel electrophoresis (19). Gel filtration was performed in buffer A on a Superose 6 column on an Amersham Pharmacia Biotech FPLC apparatus as described before (18).

Steady State Activity—NDP kinase activity was measured at 20 °C in a standard pyruvate kinase/lactate dehydrogenase coupled-enzyme assay (20) using 2 mM ATP and 0.5 mM dTDP as substrates in buffer A (see above).

Steady State Fluorescence Measurements—Fluorescence spectra of the unphosphorylated and phosphorylated enzyme were recorded on a Perkin-Elmer LS 3B fluorimeter at 20 °C. The titration experiments to determine K_{eq} were performed as described before (14), using ATP as phosphoryl donor. At half-saturation of the fluorescence change, the following relations apply: $[E] = 0.5\ [E_0] = [E\sim P] = [ADP]$ (E stands for the free enzyme, E_0 for the total enzyme, and $E\sim P$ for the phosphorylated enzyme). Designating these quantities by x, the concentration of free ATP is given by $[ATP] = [ATP_0] - x$, and from the equilibrium constant $K_{eq} = [E\sim P][ADP]/[ATP][E]$, we derive the following equation,

$$K_{eq} = x/([ATP_0] - x) \qquad \text{(Eq. 1)}$$

To ensure that NDK-B^{S122P} was unphosphorylated prior to the experiment, the enzyme was incubated at a concentration of 50 μM with 5 mM ADP, and the nucleotide was removed by gel filtration.

Stability Measurements—Experiments to determine the stability against denaturation by urea were performed in buffer A (see above) according to Ref. 21. The concentration of $MgCl_2$ was always kept in excess over the nucleotide. Native protein, as well as protein denatured in the presence of 8 M urea, were diluted into samples of buffer A containing between 0 and 8 M urea. After equilibration for 16 h at room temperature, activity was determined using the coupled enzyme assay as a measure for inactivation and reactivation. Also intrinsic tryptophan fluorescence, excited at 295 nm and measured at 340 nm, was recorded to follow denaturation. Stability toward heat was determined by incubating NDK for 8 min at temperatures between 20 and 75 °C and measuring residual activity in the coupled enzyme assay.

Rapid Kinetics—The rates of nucleotide-induced protein fluorescence changes at 20 °C in buffer A were measured with a Hi-Tech Scientific SF-51 stopped-flow apparatus equipped with a 100-watt xenon/mercury lamp and monochromator as described before (14). Intrinsic tryptophan fluorescence was excited at 295 nm and monitored through a 320-nm long-pass filter. Data were captured, stored, and analyzed using Hi-Tech software running on a PC platform. Three to ten traces were collected with the same solution, averaged, and fitted to a fitting routine in Hi-Tech software. Enzyme was mixed with at least 5-fold excess of nucleotides and concentrations are those after mixing in the stopped-flow spectrophotometer. Reaction curves were analyzed over more than 95% of the total amplitude so that at the lower limit of 5-fold excess ligand there is less than about 10% deviation from the real value of the pseudo-first order rate constant (22).

Interpretation of Kinetic Data—In our previous paper we presented evidence that the transient kinetics of the interaction of nucleotides with NDK were compatible with the following reaction scheme, which represents the donor/acceptor half-reaction for the overall reaction,

$$E + NTP \underset{}{\overset{K_1}{\Leftrightarrow}} E\text{-}NTP \overset{K_2}{\Leftrightarrow} E\sim P\text{-}NDP \overset{K_3}{\Leftrightarrow} E\sim P + NDP$$

SCHEME I

with E = NDK, $E\sim P$ = phosphorylated NDK, NTP and NDP = substrates and products, respectively, and K_i ($= k_{+i}/k_{-i}$) is the equilibrium constant of the i-th step.

In the absence of acceptor, adding excess NTP to NDK results in accumulation of phosphorylated enzyme which is accompanied by a decrease of intrinsic protein fluorescence (14, 23). The time course of this fluorescence change can be described by a single exponential. When phosphoryl transfer is much slower than nucleotide binding (*i.e.* $k_{+2} + k_{-2} \ll [ATP]\ k_{+1} + k_{-1}$), then the observed rate constant (k_{obs}) is predicted to be hyperbolically dependent on [NTP] as follows.

$$k_{obs} = K_1k_{+2}[NTP]/(1 + K_1[NTP]) \qquad \text{(Eq. 2)}$$

At low ATP concentrations such that K_1 [NTP] $\ll 1$, $k_{obs} = K_1k_{+2}$ [NTP], and the apparent second order rate constant defines K_1k_{+2}.

A similar situation holds for the reverse reaction in which addition of excess NDP to NDK∼P in the absence of NTP results in an exponential increase in fluorescence with,

$$k_{obs} = k_{-2}[NDP]/(K_3 + [NDP]) \qquad \text{(Eq. 3)}$$

and at low NDP concentrations such that $K_3 \gg$ [NDP] then $k_{obs} = k_{-2}$ [NDP]/K_3.

RESULTS

General Characterization of the Proteins

NDKs used in this study were purified to more than 95% homogeneity. Analysis with gel electrophoresis showed NDK-A and NDK-A^{S120G} migrating at 20 kDa and histidine-tagged NDK-B and NDK-B^{S122P} at 22 kDa. When analyzed by gel filtration, the elution volumes of NDK-A and NDK-A^{S120G} as well as of His-tagged NDK-B and NDK-B^{S122P} were identical, suggesting the same quarternary structure of mutant and WT proteins. The WT isoforms NDK-A and NDK-B (with the N-terminal His-tag) had an average specific activity of 150 s^{-1} at 20 °C. The mutant NDK-A^{S120G} showed a slightly reduced turnover rate of 75% of WT, whereas NDK-B^{S122P} had only 22% of the steady state activity of WT. As shown below, the low activity of the NDK-B^{S122P} is unlikely to be due to the presence of inactive protein.

The NDK-B^{S122P} appeared to be significantly less stable than the WT protein; indeed, we experienced considerable difficulty in preparing active protein in a multistep purification process. In contrast, the His-tagged protein was purified much more quickly and was active. The untagged NDK-A and NDK-A^{S120G} could be purified with high activity as shown in our previous report (9). We have previously shown that the His-tagged wild-type NDK-B has the same steady state activity and kinetic behavior as untagged NDK-B (14). All of the experiments reported here were therefore performed with the His-tagged protein.

NDK-B and NDK-B^{S122P} were checked for stability in the presence of urea and against heat denaturation. While NDK-B lost all enzymatic activity at 70 °C, NDK-B^{S122P} was inactivated at 60 °C. Unfolding of native enzyme in urea was followed by the loss of enzyme activity (not shown) and fluorescence intensity and occurred for WT NDK-B at 4 M urea and for the mutant at 3 M urea (Fig. 1, *A* and *B*). Activity and fluores-

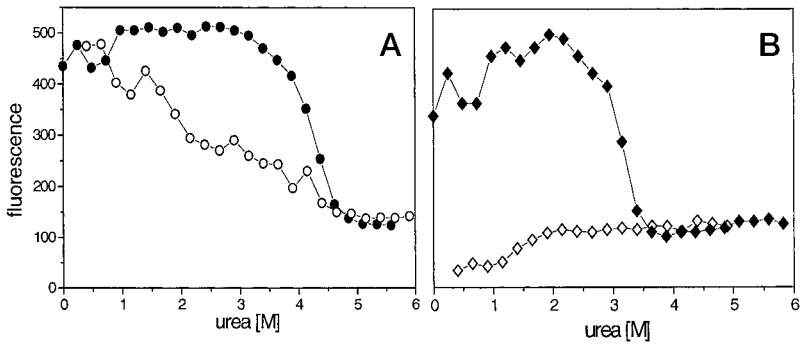

FIG. 1. **Relative fluorescence intensity of NDK-B (*A*) and NDK-B^{S122P} (*B*) in the presence of urea.** *Solid symbols* indicate the unfolding of the protein and open symbols the corresponding refolding process. 10 μM (WT NDK-B) and 17 μM (NDK-B^{S122P}) protein were equilibrated in buffer A (25 mM MOPS, pH 7.2, 100 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol) with the indicated urea concentrations at room temperature. Tryptophan fluorescence was excited at 295 nm and measured at 340 nm, at 20 °C.

cence of NDK-B, previously denatured in 8 M urea, was recovered completely below 1 M urea (Fig. 1*A*). When unfolded NDK-B^{S122P} was diluted into lower urea concentrations, no recovery of either activity or fluorescence could be observed (Fig. 1*B*). Thus, NDK-B^{S122P} is less stable than WT NDK-B. A similar reduced stability of NDK-A^{S120G} was seen in our previous study (9), denaturing at 50 °C and in 2 M urea compared with WT NDK-A at 58 °C and 3.5 M urea.

Equilibrium Titrations

Intrinsic tryptophan fluorescence of NDK-B^{S122P} was of similar intensity to that of the WT enzyme. Also, on adding ATP a 10% quench in fluorescence occurred, just as in WT, which has been shown to be due to phosphorylation of the enzyme (14, 23). The decrease in fluorescence was quite reproducible, indicating that all protein must contribute to it and little inactive protein is present.

In a titration experiment, ATP was added and the fluorescence change recorded to determine the equilibrium constant of the reaction. The K_{eq} for NDK-B^{S122P} had a value of 5 ($\pm$1) compared with 0.54 ($\pm$0.05) measured for WT NDK-B. These values can be pictured as the amount of ATP that has to be added to *e.g.* 1 μM NDK to obtain 50% phosphorylated enzyme (Equation 1), being 0.6 and 1.43 μM for mutant and WT NDK-B, respectively. No values of K_{eq} have been determined for NDK-A by this method, since the 6% fluorescence change of these proteins was too small to obtain reliable results in a titration experiment.

Transient Kinetic Studies

Exponential Phosphorylation and Dephosphorylation Reactions—Nucleotide-induced fluorescence transients of the mutant proteins NDK-B^{S122P} and NDK-A^{S120G} were measured using the approach that was established in our previous study of the WT proteins (14). Rapid mixing of 0.5 μM NDK-B with 15 μM ATP resulted in a 9% quench in fluorescence, which could be described by a single-exponential process with a rate constant of 124 s^{-1} (Fig. 2*A*). In contrast, the fluorescence of the mutant NDK-B^{S122P} was quenched by 10.5% at a lower rate of 41 s^{-1} when mixed with 30 μM ATP (Fig. 2*B*). Phosphorylated enzyme was prepared as described under "Materials and Methods," and rapid mixing of 0.5 μM NDK-B~P with 5 μM ADP resulted in a 6% increase in fluorescence at a rate of 101 s^{-1} (Fig. 2*C*). Phosphorylated mutant enzyme, NDK-B^{S122P}~P, was dephosphorylated by 5 μM ADP at a much lower rate of 1.5 s^{-1}, with a larger increase in fluorescence of 8% (Fig. 2*D*). The larger amplitudes observed for the mutant protein are compatible with the larger value of K_{eq} for the overall reaction.

Wild-type NDK-B Reactions—We previously characterized the reaction of the WT protein with ATP, GTP, ADP, and GDP over a range of concentrations (14). The observed rate constants, k_{obs}, were linearly dependent upon nucleotide concentration over the accessible range, and apparent second order rate constants were obtained from linear fits to plots of k_{obs} *versus* nucleotide (Table I). The data were interpreted in terms of the three-step model for the reaction as shown in Scheme I.

Mutant NDK-B^{S122P} Phosphorylation—The dependence of the rate of phosphorylation of NDK-B^{S122P} on nucleotide concentration was studied with ATP and GTP, and the data are plotted in Fig. 3A. The data show that the observed rate constant for phosphorylation by ATP was linearly dependent upon ATP concentration for both proteins, but the apparent second order rate constant, K_1k_{+2} (Eq. 2), was 5-fold lower for NDK-B^{S122P} (Table I, A). In contrast, the observed rate of the reaction with GTP appeared to saturate at high concentrations. The data could be described by a hyperbola, as predicted from Equation 2, giving an apparent second order rate constant 4-fold slower than for WT and a value of 196 s^{-1} for k_{+2} (the maximum value of k_{obs}) and) 53 μM for $1/K_1$ (or $K_{0.5}$ = the concentration of GTP required to give $k_{obs} = k_{max}/2$) (Table II). The value of k_{+2} for the WT protein is thought to be too fast to measure by the stopped flow method, but we did previously observe hyperbolic dependence of the reaction when using ATPγS or GTPγS (14). We reported that the values of k_{obs} for phosphorylation by ATPγS or GTPγS were reduced about 1000-fold compared with ATP and GTP, and we determined both k_{+2} and $1/K_1$ (Table II, A). From our previous experiments we argued that nucleotide affinity was not much affected by the thio-modification, and therefore K_1 is similar to that of the equivalent unmodified nucleotide (14). With NDK-B^{S122P} and thio-modified substrates the reaction was too slow to follow even with nucleotide concentrations up to 1 mM, and the fluorescence signal disappeared in the background due to the absorbance of the nucleotide. This is expected if the value of k_{+2} is also 1000-fold reduced for the thiophosphoryl transfer.

Mutant NDK-B^{S122P} Dephosphorylation—Studies of the dephosphorylation reaction of NDK-B^{S122P} by either ADP or GDP produced hyperbolic plots (Fig. 3*B*) with apparent second order rate constants (k_{-2}/K_3) reduced more than 30-fold compared with WT in each case. The values of k_{-2} (k_{max}) and K_3 ($K_{0.5}$) are listed in Table II, B. In contrast to the mutant, the WT protein showed no evidence of deviation from a linear dependence on NDP concentration. This suggests that the major effect of the mutation is to slow the phosphoryl transfer steps in the direction of both phosphorylation and dephosphorylation.

We repeated these measurements with NDK-B^{S122P} for a range of NDP and dNDP nucleotides and the results are summarized in Table II. With the exception of CDP and dCDP, the data showed a hyperbolic dependence on NDP concentration and estimates of k_{-2} and K_3 were obtained. For CDP and dCDP

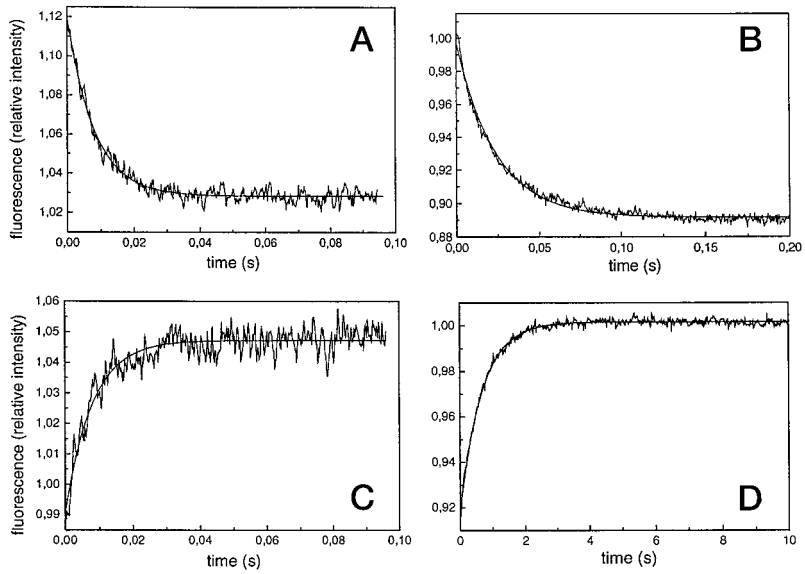

FIG. 2. **Time course of nucleotide induced fluorescence changes of 0.5 μM enzyme excited at 295 nm and measured above 320 nm.** Phosphorylation of WT NDK-B with 15 μM ATP occurred at a rate k_{obs} of 124 s^{-1} (± 7) and an amplitude of 9% (*A*) (data from Ref. 14), phosphorylation of NDK-B^{S122P} with 30 μM ATP at k_{obs} = 41 s^{-1} (±0.5) and an amplitude of 10.5% (*B*). Dephosphorylation of WT NDK-B~P with 5 μM ADP had a k_{obs} = 119 s^{-1} (±9) and an amplitude of 6% (C) (data from Ref. 14), and that of NDK-B^{S122P}~P at the same ADP concentration had a k_{obs} = 1.5 s^{-1} (±0.05) and an amplitude of 8% (*D*). All measurements were performed at 20 °C in buffer A.

TABLE I
Apparent second order rate constants (10^{-6} M^{-1} s^{-1}) for phosphorylation (A; K_1k_{+2}) and dephosphorylation (B; k_{-2}/K_3) for NDK-B and NDK-B^{S122P}

	A: Phosphorylation	
	NDK-B[a]	NDK-B^{S122P}
ATP	7.9 (±0.5)	1.4 (±0.3)
GTP	12.2 (±0.5)	3.2 (±0.8)
	B: Dephosphorylation	
	NDK-B~P[a]	NDK-B^{S122P}~P
ADP	17.9 (±1)	0.55 (±0.16)
GDP	29.1 (±2.4)	0.8 (±0.2)
UDP	3.8 (±0.4)	0.04 (±0.003)
CDP	1.3 (±0.04)	0.01 (±0.001)

[a] Data from Ref. 14.

the data appeared linearly dependent upon NDP concentration up to at least 2 mM.

Thiophosphoryl Transfer Reactions—In our previous study, thiophosphorylated WT protein was mixed with a wide range of NDPs and a hyperbolic dependence on NDP concentration appeared. This allowed estimates of k_{-2} and K_3 for the same series of substrates (Table II). We previously argued that the value of K_3 (the affinity of NDP for the protein) may be similar for NDK~P and NDK~thioP. The values of K_3 for both NDK-B^{S122P}~P and NDK-B~thioP are listed in Table II, B. They differ by a factor of 0.3–4 for each NDP and 0.2–3 for each dNDP. We conclude that the mutation S122P primarily affects the rate of phosphoryl transfer (k_{-2}) and not the affinity of NDP for the protein (K_3).

This conclusion is supported by the results for GTP and GTPγS phosphorylating NDK-B and NDK-B^{S122P}. The values of $1/K_1$ with GTP or GTPγS and WT were 25 and 36 μM, respectively, and for the mutant, $1/K_1$ with GTP had a value of 53 μM. Thus, although the observed rates of phosphorylation and thiophosphorylation of NDK-B (controlled by K_1k_{+2}) differ by more than 300-fold, K_1 is relatively invariant.

Equilibrium Constants—For NDK-B^{S122P}, the phosphorylation reaction with ATP and GTP is 4–5-fold slower than for the WT enzyme (Table I, A). The dephosphorylation reaction is even more affected, being 40-fold reduced with ADP and GDP and 100–200-fold with the other nucleoside diphosphates (Table I, B). As K_{eq} is defined by the ratio of k_{phos}/k_{dephos}, its value lies between 4.3 and 4.4 for NDK-B^{S122P}, and between 0.44 and 0.42 for NDK-B, when using the apparent second order rate

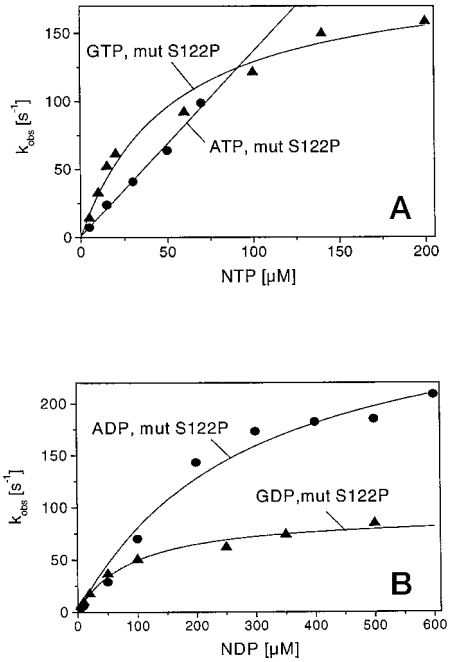

FIG. 3. *A*, observed rate constants, k_{obs}, for phosphorylation of NDK-B^{S122P} with ATP and GTP. *B*, dephosphorylation reaction of previously phosphorylated NDK-B^{S122P} with ADP and GDP. Conditions are the same as in Fig. 2.

constants for the pairs ATP/ADP and GTP/GDP. This value is in good agreement with the values of K_{eq} of 0.54 for NDK-B and of 5 for NDK-B^{S122P} obtained by equilibrium titration with ATP.

In summary, when comparing with WT NDK-B, the affinity of NDK-B^{S122P} for its substrates and the phosphorylation reaction seem to be only slightly affected, whereas the initial and the maximal rates for the dephosphorylation reaction are reduced significantly.

Phosphorylation of NDK-A and NDK-A^{S120G}

Our earlier studies of NDK-A were repeated with the NDK-A^{S120G} mutant protein. We measured the rates of phosphorylation by ATP, GTP, and ATPγS and of dephosphorylation by ADP and GDP and compared them to WT NDK-A (data on WT NDK-A from Ref. 14). For NDK-A^{S120G}, the fluorescence signal showed an amplitude change of −6% for phosphorylation and

TABLE II
Maximal rate (k_{max}) and substrate concentration at which half-k_{max} is reached ($K_{0.5}$), determined with NDK-B (only thionucleotides (data from Ref. 14)) and with NDK-B^{S122P}

	A: Phosphorylation		
	NDK-B (NTPγS), $K_{0.5}$[a]	NDK-B^{S122P}	
		$K_{0.5}$	k_{max}
	μM	μM	s^{-1}
ATP	145 (±15)	*Linear to 100 μM*	*≫120*
GTP	36 (±4)	53 (±10)	196 (±14)

	B: Dephosphorylation		
	NDK-B~thioP, $K_{0.5}$[a]	NDK-B^{S122P}~P	
		$K_{0.5}$	k_{max}
	μM	μM	s^{-1}
ADP	1050 (±650)	310 (±35)	285 (±73)
GDP	165 (±5)	88 (±15)	93 (±5)
UDP	2320 (±325)	6404 (±409)	343 (±18)
CDP	3249 (±815)	*Linear to 2 mM*	*≫20*
dADP	1300 (±400)	520 (±100)	40 (±4)
dGDP	192 (±14)	108 (±18)	21 (±1)
dUDP	612 (±133)	2975 (±430)	27 (±3)
dCDP	2130 (±1372)	*Linear to 3 mM*	*>4*
dTDP	900 (±590)	1684 (±330)	75 (±8)

[a] Data from Ref. 14.

TABLE III
Apparent second order rate constants (× 10^{-6} M^{-1} s^{-1}) for phosphorylation (A; K_1k_{+2}) and dephosphorylation (B; k_{-2}/K_3) of NDK-A and NDK-A^{S120G}

	A: Phosphorylation	
	NDK-A[a]	NDK-A^{S120G}
ATP	5.2 (±1)	1.6 (±0.3)
GTP	9.2 (±2.2)	3.0 (±0.7)
ATPγS	k_{+2}: 2.9 (±0.07) s^{-1} $1/K_1$: 198 (±12) μM	k_{+2}: 1.1 (±0.03) s^{-1} $1/K_1$: 113 (±9) μM

	B: Dephosphorylation	
	NDK-A~P[a]	NDK-A^{S120G}~P
ADP	19 (±10)	13.4 (±4.8)
GDP	27 (±5)	21.7 (±1.1)

[a] Data from Ref. 14.

+4% for dephosphorylation, similar to WT NDK-A. As suggested from steady state measurements (8, 9, 24), no severe effect on the rates of the reaction could be observed. Compared with WT, all observed rate constants were within a factor of 3 for phosphorylation and within a factor of 1.5 for dephosphorylation (Table III). The ratios of k_{obs} values in the phosphorylation and dephosphorylation reaction suggest a decrease in the K_{eq} for the reaction from about 0.3 for the wild type to 0.13 for the mutant. However, because of the errors on the values of k_{obs}, this change in K_{eq} is not well defined.

DISCUSSION

Human NDK-A and NDK-B and the two mutants NDK-A^{S120G} and NDK-B^{S122P} found in tumor cells were expressed in *E. coli* and purified. Both mutants had a lower resistance to denaturation by urea and by heat compared with the corresponding wild types. Refolding of NDK-A^{S120G} was severely disturbed leading to molten globule-like structures (9). It was pointed out before that accumulation of incompletely folded NDK-A^{S120G} would be harmful to the cell and thus may eventually initiate transformation (9). From available crystal structures of human NDK-B (25, 26), it was supposed (9) that the highly conserved Ser^{120}, which is not exposed at the surface, stabilizes the structure by forming internal ionic interactions. In the S120G mutant these interactions are absent, which would eventually affect the stability of the whole hexamer.

NDK-B^{S122P} does not refold after denaturation in urea, suggesting that *in vivo* unfolded protein could also accumulate for this mutant. The high-resolution crystal structure of NDK-B (25, 26) indicates that Ser^{122}, which is a highly conserved residue in all NDKs, is located at the start of an α-helical region which covers the top face of a β-sheet. This helix, together with another α-helix, forms a cleft extending the nucleotide binding cavity. Mutation from serine to proline may be expected to disrupt the helix at this point and could thus reduce stability. In addition to reduced stability, the cellular effect of the S122P mutation could also be caused by the loss of a potential phosphorylation site, which might be relevant for a yet unknown role of NDK. The Ser^{122} residue has previously been proposed to be a putative site of autophosphorylation (27) or being targeted by casein kinase (28). Also, the DNA binding role of NDK-B (29, 30) may be affected by the S122P mutation, but more detailed and quantitative studies of DNA binding to NDK-B and NDK-B^{S122P} would be needed to clarify this issue.

NDK-A^{S120G} showed relatively minor alterations in its enzymatic properties; a 3-fold reduction in phosphorylation rates and little effect on dephosphorylation gives an approximately 3-fold decrease in K_{eq}. A much greater effect was seen for NDK-B^{S122P}. Our present work suggests that the rate of NDK-B^{S122P} phosphorylation is relatively little affected (up to 5-fold reduced), whereas the dephosphorylation reaction reveals a more dramatic impact of the S122P mutation: initial rates are 40–200-fold reduced. Since the affinities for the substrates are altered less than 3-fold, this reduction is primarily in the phosphoryl transfer rates. Due to the slower dephosphorylation the equilibrium constant changed from 0.5 (for WT) to 5, favoring the phosphorylated state of the mutant enzyme.

The effect of changes in enzymic properties on the cell will depend upon the overall balance of nucleoside phosphoryl transfer reactions taking place in the cell. If the cell is in steady state and the role of the NDK proteins is the rapid equilibration of NTPs and NDPs, then we can consider the effects of a change in rate of phosphoryl transfer and a change in K_{eq}. If the rate of phosphoryl transfer remains fast enough to rapidly equilibrate all NDP/NTP (which probably is also true of NDK-A^{S120G}) then the relation between the phosphorylation state of the enzyme and K_{eq} is given by $E\sim P/E = K_{eq}\cdot ATP/ADP$. Assuming an ATP/ADP ratio of the order of 10 to 100, then the $E\sim P/E$ ratio will be 3 to 30 for NDK-A, 1.3 to 13 for NDK-A^{S120G}, 5 to 50 for NDK-B, and 50 to 500 for NDK-B^{S122P}. Thus, the fraction of nonphosphorylated NDK-A is changed due to an altered K_{eq} from 3–25% (for WT) to 7–43% for NDK-A^{S120G}. If the phosphorylation state of the protein is sensed by another protein this effect could be significant. In the case of NDK-B, although the change in K_{eq} is greater, it is in the direction of more phospho-enzyme; thus, the protein will be predominantly phosphorylated for both the WT and the mutant under all conditions. Therefore, any role of NDK-B as a sensor of the metabolic state (ATP/ADP ratio) of the cell or in buffering the NTP concentration is unlikely to be affected by the change in K_{eq}.

The 5-fold decrease in the rate of NDK-B^{S122P} phosphorylation is expected to have no significant effect on the net rate of phosphoryl transfer among the nucleotides in the cell, but the 40–200-fold reduction in the rate of dephosphorylation may affect the cell particularly under conditions of high NTP turnover which could occur locally or globally in a cell. This is particularly true for CDP, UDP, and the corresponding dNDPs, since these rates are most affected. As the rates are already relatively slow for these nucleotides compared with those of ADP and GDP in the WT, it is possible that the concentrations of CTP, UTP, dCTP, and dUTP become limiting under some

conditions. It has been speculated that NDK is the provider of GTP for the cell in a variety of processes such as protein synthesis (31, 32), signal transduction pathways (33, 34, 35), or microtubule-based motility (36, 37). The maximal rate of GDP phosphorylation through NDK-B would be reduced from 4800 s^{-1} (14) to about 100 s^{-1}, and thus the flux from ATP to GTP would be greatly affected especially under high energy consumption. This may upset the cell signaling pathways mediated by GTP.

In summary, the change in K_{eq} could be significant for NDK-A^{S120A}, and the change in the rate of dephosphorylation of the enzyme could be significant for NDK-B^{S122P}. In the cell, however, any such effects may be influenced by changes in expression levels of mutant or wild-type NDK-A and NDK-B, especially when considering the formation of mixed hexameric species of NDK which do occur *in vivo* (38). The effect of the mutations on mixed NDK-A/NDK-B hexamers has not been studied in this work.

It has been speculated that side chain phosphorylation modulates a function of NDK-B, possibly the transcription factor activity (30), protein kinase activity (28, 39), or even the oligomeric structure of the enzyme (6, 40). But so far it is not known whether the His^{118}-phosphorylated NDK is simply an intermediate in the catalytic reaction pathway or whether it has yet another role (41), which would be affected and possibly enhanced by the S122P mutation. From the crystal structure, the changes in catalytic activity cannot be explained easily for either S120G or S122P; both serines are separated from the catalytic histidin H118 by another residue and do not participate in nucleotide binding (25, 26). The bigger effect of the S122P mutation may be due to the possible distortion of a helical element as discussed above. However, it is not obvious how this alteration should favor the phospho-enzyme state of NDK-B^{S122P}. The transforming effect of NDK-B^{S122P} on the cell could originate from either the reduced stability (as is probably the case for NDK-A^{S120G}), from the missing phosphorylation site for a yet unknown function, or from the altered activity as seen especially in the greatly reduced NDP phosphorylation rate.

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ENZYMOLOGY:

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