The Catalytic Mechanism of Nucleoside Diphosphate Kinases

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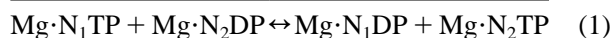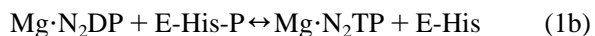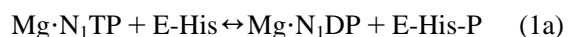
Nucleoside diphosphate kinases catalyze the reversible transfer of the γ phosphate of nucleoside triphosphates to nucleoside diphosphates. This minireview presents recent advances in understanding the reaction mechanism using steady-state and fast kinetic studies, X-ray crystallography, and site-directed mutagenesis. We also briefly discuss the physiological relevance of *in vitro* studies.

INTRODUCTION

It is now well established that the NDP kinase reaction proceeds via a covalent intermediate, the enzyme being transiently phosphorylated at a histidine residue:

$$Mg \cdot N_1TP + E\text{-His} \leftrightarrow Mg \cdot N_1DP + E\text{-His-P} \quad (1a)$$

$$Mg \cdot N_2DP + E\text{-His-P} \leftrightarrow Mg \cdot N_2TP + E\text{-His} \quad (1b)$$

$$Mg \cdot N_1TP + Mg \cdot N_2DP \leftrightarrow Mg \cdot N_1DP + Mg \cdot N_2TP \quad (1)$$

where NDP and NTP are nucleoside (or 2′-deoxynucleoside) diphosphate and triphosphate, respectively. NDP kinases show remarkable sequence conservation and have identical active-site residues. The crystal structures of the various NDP kinases are also very similar. It is, therefore, likely that all NDP kinases have identical mechanisms. Recently, sequences encoding NDP kinaselike proteins (nm23-H5 to H8) and NDP kinaselike modules in the intermediate chain of dynein (Ogawa *et al.*, 1996), have been identified. Detailed structural and biochemical studies of these proteins have not yet been performed and, therefore, will not be included in this discussion.

The older results were summarized in a review by Parks and Agarwal (1973). A short overview of the mechanism of NDP kinases is given in Lascu *et al.* (1996). The role of phosphoryl intermediates in catalysis has also been reviewed (Frey, 1992; Johnson and Barford, 1993). Other general reviews on phosphoryl transfer may also be useful (Matte *et al.*, 1998; Mildvan, 1997; Knowles, 1980).

For the sake of simplicity, the residue numbers given here are those for the human NDP kinases A and B.

PRACTICAL ASPECTS OF ACTIVITY MEASUREMENT

A variety of enzyme assays has been developed, but only a few are in current use. The most convenient is the spectrophotometric assay, with pyruvate kinase and lactate dehydrogenase reactions used as indicator reactions. The background rate is due to the contamination of the pyruvate kinase with NDP kinase and to NTPase activity in the sample. ATP or GTP is used as a donor (as the product, ADP or GDP is a good substrate for pyruvate kinase), whereas the acceptor diphosphate used should be a poor substrate for pyru-

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vate kinase. 8-Bromoinosine 5′-diphosphate (Kezdi *et al.*, 1976) and TDP are suitable acceptors. This restriction on the use of nucleotides as acceptors does not apply to the isotopic (Pedersen and Cattarall, 1979) and HPLC assays (Lambeth and Muhone, 1993), but such experiments are tedious to perform. However, these methods do make it possible to perform assays with several competing substrates simultaneously, as has been done, for example, for ribonucleotide reductase (Hendricks and Mathews, 1997). A modified version of this method has been used by Biondi *et al.* (1995), but in single-turnover conditions. Incubation of phosphorylated NDP kinase with a mixture of nucleoside diphosphates has been used in the analysis of substrate specificity. Multiple rounds of catalysis can be avoided by Cibacron Blue 3G-A to the reaction mixture. This dye binds to the free form of NDP kinase, but not to the phosphorylated form (Lascu *et al.*, 1983). Unfortunately, no mathematical model is available for this elegant experiment and the nature of the biochemical information obtained is not clear.

A coupled assay has been described, using the peroxidase reaction as the indicator (Lascu *et al.*, 1993). This flexible system can also be used to stain the gel after electrophoresis (Timmons *et al.*, 1995). For maximum sensitivity, a luminometric assay was developed (Karamohamed *et al.*, 1999) in which the ATP formed by the NDP kinase reaction is used as a substrate for luciferase.

The initial rate should be measured in all cases. Rates should be given instead of the photographs of nucleotide separation by thin-layer chromatography that are often found in published papers. The results described in several papers cannot be quoted because mutants, for example, are merely "active." In other cases, the reaction appears on inspection to be at equilibrium. It is also important not to use the *amount* of phosphate incorporated into NDP kinase as an *activity* measurement. This is a single-turnover reaction in which steady-state kinetic equations cannot be used. Because of the high turnover of NDP kinases, even an enzyme with low specific activity appears to be phosphorylated as a fully active enzyme.

Finally, the precision of specific activity measurements also depends on the precision of protein concentration determination. Large differences have been found between extinction coefficients calculated from amino acid composition (Gill and von Hippel, 1989) and those calculated using an absolute method—amino acid analysis. This difference is largest for bacterial

NDP kinases devoid of tryptophan (Anna Giartosio and Donatella Barra, personal communication, 1997).

STEADY-STATE KINETIC ANALYSIS

The yeast enzyme was thoroughly analyzed by Cleland (Garces and Cleland, 1969), who demonstrated that it followed a ping-pong Bi-Bi mechanism. Both substrates appear to be active as complexes with Mg^{2+} ions. The ping-pong mechanism results in inhibition by an excess of either substrate, with the formation of abortive complexes. The nucleoside diphosphate binds to the free enzyme, whereas the nucleoside triphosphate binds, albeit with a lower affinity, to the phosphorylated enzyme. The detailed mechanism is summarized in Scheme 1. The kinetic equations have been reported by Garces and Cleland, 1969, or may be derived by the net flux method of Cleland (1977). Reactions 3 and 6 should be considered irreversible in steady-state measurements, but not automatically so in fast kinetic studies.

Inhibition by excess NDP is difficult to avoid because the value of the $K_{m,NDP}$ is similar that for the $K_{i,NDP}$. It is time-consuming and difficult to obtain the "true" values for K_m and k_{cat}. k_{cat} may be obtained with greater precision by varying the concentration of both nucleoside di- and triphosphates in a constant ratio (Segel, 1993). There is still a small error on this parameter. V_{max} values are typically between 300 and 2000 units/mg (k_{cat} about 100–600 s^{-1}), whereas the $K_{m,ATP}$ is about 500 μM and the $K_{m,TDP}$ about 250 μM, with NDP kinases from several sources. The actual values depend on the nature of the nucleotides used. Tetrameric and hexameric NDP kinases display similar kinetic parameters. The apparent kinetic parameters, measured at a fixed concentration of one substrate, are a function of that concentration and can be calculated using Eq. 2. They are far from the true values. The K_m and K_d also differ greatly, by a factor of about 100 for ATP.

For practical reasons, it is easier to measure k_{cat}/K_m because this is equal to $k_{cat,app}/K_{m,app}$. When working with substrates that have a high K_m, this parameter is simply the slope of the linear plot of k_{cat} against substrate concentration [if (s) $\ll K_m$]. $k_{cat}/K_{m,NDP}$ may also be measured as the first-order rate constant of NDP disappearance with excess ATP. Inhibition by excess ATP should be included in this analysis.

Scheme 1.
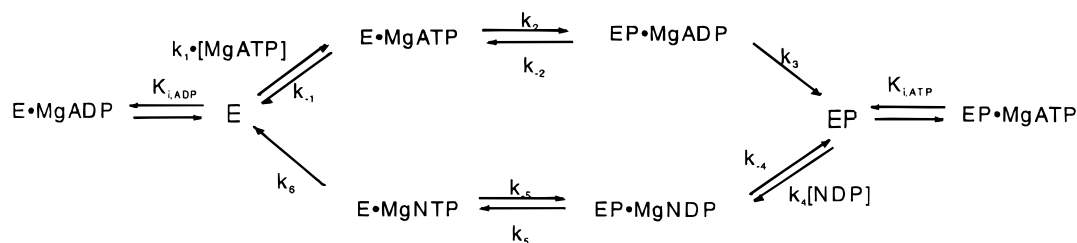
SUBSTRATE SPECIFICITY

NDP kinases are considered "nonspecific" with respect to the base moiety of the donor and acceptor nucleotides. However, differences have been found in steady-state experiments with various nucleotides. Guanine nucleotides are the best substrates, whereas cytosine nucleotides are the poorest, in terms of both k_{cat} and K_m. However, analysis with an extensive series of nucleotides has never been done. It is straightforward to carry out fast kinetic analysis of the half-reactions (1a) and (1b) separately. This is easily done with NDP kinases because the fluorescence of the tryptophan 133 is quenched by phosphorylation (Deville-Bonne *et al.*, 1996). Substrate binding has no effect on protein fluorescence. Free enzyme and E·NTP complex, cannot be distinguished; neither can EP and EP·NDP. The ratio of the second-order rate constants with guanine nucleotides (the best substrates) and cytosine nucleotides (the poorest substrates) is from 17 to 22 for NDP and NTP, up to 50 for 2′-dNTPs (Schaertl *et al.*, 1998) and 120 for 2′,3′-ddNTPs (Schneider *et al.*, 1998b). The chemical step (reaction 5 in Scheme 1) probably becomes rate limiting with the ddNTP (see below) and discrimination seems to occur at the chemical step. The better interaction with guanine nucleotides may be due to the base interaction with the C-terminal glutamate of the neighboring subunit. No such interaction occurs, however, in the tetrameric NDP kinase from *Myxococcus*.

NDP kinases accept 2′-deoxynucleotides as substrates, although the catalytic efficiency is slightly lower. Concerning the phosphorylation of dNDPs *in vivo*, whatever the donor, k_{cat}/K_m is the most important parameter because concentrations are well below the K_m. The same is also true for poor nucleotide acceptors, such the diphosphates of the anti-AIDS nucleosides (see Schneider *et al.*, 2000a, 2000b).

All modifications to the phosphate chain lead to large decreases in catalytic efficiency. The replacement of one oxygen of phosphates α and β by sulfur generates diastereoisomers. Only adenosine 5′-0-1-(thio)triphosphate S (Eckstein and Goody, 1976; Burgers and Eckstein, 1978) and adenosine 5′-0-2-(thio)triphosphate R (Cohn, 1982) diastereoisomers are substrates for NDP kinase. The absolute configuration of the isomers is known. When placed in the structure of the NDP kinase–ADP–magnesium complex, the metal ion can bind oxygen atoms. Sulfur has a much lower tendency to complex magnesium, accounting for enzyme stereospecificity. Adenosine 5′-0-(3-thiotriphosphate) is a poor substrate, decreasing k_{cat} by a factor of about 300. This decrease in catalytic efficiency may be due to the sulfur atom being larger than that of oxygen. This results in the sulfur atom being poorly accommodated in the active site in the transition state (Admiraal *et al.*, 1999). Alternatively, the partial positive charge on the γ phosphate may be lower because sulfur is less electronegative than oxygen. This renders the nucleophilic attack of the nitrogen imidazole (reaction 1a) and of the β-oxygen of NDP (reaction 1b) more difficult.

Adenosine 5′-sulfatopyrophosphate has been shown to be a weak substrate for NDP kinase. The sulfuryl transfer was 1000 times slower than phosphoryl transfer, but the K_m was similar to that of ATP. The sulfurylated NDP kinase was isolated and hydrolysis of the sulfurylated histidine was found to be much faster than that of the phosphorylated histidine (Peliska *et al.*, 1991).

The H118G mutant was inactive, but imidazole was able to bind in place of the missing histidine side chain. The resulting protein catalyzed imidazole phosphorylation by ATP (Admiraal *et al.*, 1999).

ROLE OF DIVALENT METAL IONS IN CATALYSIS

Divalent metal ions have been shown to be essential in phosphoryl transfer reactions (Knowles, 1980).

Interaction with the metal ion decreases electrostatic repulsion and stabilizes the transition state by neutralizing the developing charges. NDP kinase is no exception to this rule. In steady-state experiments, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, and Zn^{2+} ions are active, in decreasing order of efficiency (Parks and Agarwal, 1973). Rapid kinetic studies have also demonstrated the importance of Mg^{2+} ions (Colomb *et al.*, 1972b). In crystal structures of NDP kinase with nucleotides, Mg^{2+} ions appear to be tridentate (Admiraal *et al.*, 1999; Xu *et al.*, 1997). The metal ion does not interact directly with the protein. However, water molecules mediate interaction with Glu54 and Asp121. The mutation of Asp121 to an Asn inactivates the NDP kinase of *Drosophila* (Timmons *et al.*, 1995).

It has been reported that the NDP kinase of *Myxococcus* can be phosphorylated in the presence of EDTA and without the addition of magnesium ions (Munoz-Dorado *et al.*, 1990). The authors used this as evidence that reaction (1a) occurred in the absence of divalent cations. In contrast, they found that the full phosphotransfer reaction (1) was divalent cation-dependent. They concluded that there was a fundamental difference between reactions (1a) and (1b).

This conclusion generates two key questions: (1) Do reactions (1a) and (1b) have different chemical mechanisms? (2) Can reaction (1a) occur in the absence of divalent metal ions?

The answer to the first question is "no, they do not." Different mechanisms for reactions (1a) and (1b) would not be consistent with the principle of microscopic reversibility. This fundamental principle requires that "the transitions between any two states take place with equal frequency in either direction, at equilibrium" (Fersht, 1999). In other words, the transition states of reactions (1a) and (1b) must be identical, as reaction (1b) is chemically identical to the reverse of reaction (1a). The dependence on metal ions of the two reactions must be identical. Structural studies have shown that the donor NTP and the acceptor NDP bind at exactly the same binding site.

The second question is perhaps not precise enough. It is probably more appropriate to ask about the residual reaction rate in the total absence of metal ions. For the experimentalist, the addition of EDTA is by no means equivalent to the absence of magnesium ions. Trace amounts of Mg^{2+} and other divalent ions contaminate the NDP kinase preparation, the chemicals, and the glassware. In addition, the dissociation constant of the MgEDTA complex is as high as 1 μM. The magnesium concentration is certainly very low

and the NDP kinase reaction is undetectable under conditions of multiple rounds of catalysis. However, enzyme phosphorylation may be detected in single-turnover conditions. This reaction is very slow on the time-scale of NDP kinase turnover (phosphorylation is completed in minutes whereas turnover is higher than 100 s^{-1}). Its rate is so low that the contribution of phosphorylation independent of metal ions is irrelevant. The careful elimination of all possible traces of contamination was found to abolish phosphorylation with [γ-^{32}P]ATP completely (Erent *et al.*, 1995; Polosina *et al.*, 1998). It may, however, still be possible to detect some phosphorylation using a more sensitive analytical method or a longer incubation time.

As for other phosphotransferases, the activity of NDP kinase is inhibited by high concentrations of Mg^{2+}. This is due to the formation of an ATP complex with two Mg^{2+} ions. However, direct interaction with the enzyme cannot be excluded.

INHIBITORS OF NDP KINASE ACTIVITY

Any alternative substrate acts as a competitive inhibitor. UDP is used at high concentration (10 mM) as a competitive inhibitor for GDP as substrate, for example. The NDP are also dead-end inhibitors. Nucleotides that bind but do not transfer the phosphoryl act as inhibitors. Such nucleotides include the nucleoside monophosphates (Parks and Agarwal, 1973), cAMP (Strelkov *et al.*, 1995), and cAMP analogs (Anciaux *et al.*, 1997). The crystal structures of the *Myxococcus* NDP kinase–cAMP complex (Strelkov *et al.*, 1995) and beef NDP kinase–cGMP (Abdulaev *et al.*, 1998) have been determined and indicate that the nucleotides bind to the active site. 3'-Phosphorylated nucleotides inhibit NDP kinase. 5'-Phosphoadenosine 3'-phosphate has a K_d of 100 μM, whereas adenosine 3'-phosphate 5'-phosphosulfate binds with a K_d of 10 μM. The crystal structure of the complexes formed with these molecules shows that the 3'-phosphate is located close to the γ-phosphate in the transition state (Schneider *et al.*, 1998b). The nucleoside triphosphate analogs 5'-adenylyl-β,γ-imidodiphosphate and 5'-adenylyl-β,γ-methylenediphosphate do not bind with high affinity to NDP kinase but are very strong inhibitors of a variety of other phosphotransferases. The oxygen bridge between the β- and γ-phosphates makes a hydrogen bond with the 3'-ribose hydroxyl group when the nucleotides are bound to NDP kinases (see below). This interaction cannot occur with the modi-

fied nucleotides. The substitution of a methylene for the oxygen bridging the α- and β-phosphates is well tolerated by NDP kinase. 5′-Adenosyl-α,β-methylene-diphosphate is a good phosphoryl acceptor (Colomb *et al.*, 1972a).

Incubating NDP kinase with ADP, sodium fluoride, and aluminium chloride generates the ternary complex in the crystal (Xu *et al.*, 1997). Surprisingly, however, this is not a good system for specifically inhibiting NDP kinase activity (Xu, 1998). The inhibition observed is weak and depends on incubation time and the order in which the reagents were mixed. Aluminium trifluoride is probably a minor species in solution. In crystallization conditions (high concentrations and long incubation time), the enzyme may bind this species, which is complementary to the active site. On the other hand, vanadate is not an inhibitor of NDP kinase activity (I. Lascu, unpublished results, 1993).

Several classes of anionic dye have been shown to bind to the active site of NDP kinase. The antiallergic drug, chromoglycate, inhibits NDP kinase activity with a K_i of 2 mM (Hemmerich *et al.*, 1992). The iodinated xanthene dye, Rose Bengal, has a K_d of 0.9 μM for the free enzyme and a markedly lower affinity for the phosphorylated enzyme (Lascu *et al.*, 1986). This dye, as the cognate erythrosine, has been used as a competitive probe for the binding of nucleotides to NDP kinase (Robinson *et al.*, 1981; Lascu *et al.*, 1983). Cibacron Blue 3G-A, the chromophore of the widely used Blue Sepharose, binds tightly to the free enzyme (K_i 0.3 μM), but has no affinity for the phosphorylated enzyme (Lascu *et al.*, 1983). Procion Blue, which has a very reactive dichlotriazinyl moiety, has been used for the synthesis of a spin-labeled inhibitor (Porumb *et al.*, 1984). Measurements of the binding of this derivative by electron spin resonance, and of Cibacron Blue binding by polarography, may be useful for some applications because these two methods detect the free dye. The dyes bind the active site with high affinity despite their lack of structural similarity to the substrate. The presence of anionic and hydrophobic groups is sufficient for tight binding. Desdanine [*trans*-3-(1-pyrrolin-2-yl)-acrylamide] irreversibly inhibits the NDP kinase from *E. coli* (Saeki *et al.*, 1974), but with low affinity. Agou *et al.* (2000), studied the binding of and inhibition of activity by oligonucleotides. Finally, polyclonal anti-NDP kinase antibody from frog (Yi *et al.*, 1996) and monoclonal anti-*Dictyostelium* NDP kinase (cytosolic) antibody inhibit NDP kinase activity (M. Véron, personal communication).

No strong and specific inhibitor of NDP kinase activity has yet been identified. All the inhibitors discussed above are competitive in nature. Their efficiency, therefore, decreases if high substrate concentrations are used. It would be useful to have available a noncompetitive, or irreversible inhibitor. The known strong inhibitors, such as Cibacron Blue and histidine-modifying reagents (diethyl pyrocarbonate) are too nonspecific for use, for example, in cell biology experiments.

THE PHOSPHORYLATED INTERMEDIATE

The covalent phosphoenzyme was first identified in the mid-1960s by chemical analysis. Later studies provided several lines of evidence demonstrating that the phosphoenzyme is a true intermediate in the catalytic cycle and not a by-product. Sheu *et al.*, (1979) showed that the configuration of the phosphoryl group was retained during the NDP kinase reaction. This corresponds to an inversion of configuration for each of the partial reactions, (1a) and (1b). Fast kinetic studies showed that the phosphorylation and dephosphorylation steps are as fast as the rate measured by steady-state experiments (Walinder *et al.*, 1969; Schaertl *et al.*, 1998).

The location of the active-site histidine was identified by direct protein sequencing (Gilles *et al.*, 1991), site-directed mutagenesis, and X-ray crystallography (Dumas *et al.*, 1992). Histidine replacement can be used to prepare completely inactive NDP kinase that is native, binds nucleotides, and correctly assembles into oligomers (Dumas *et al.*, 1992; Schneider *et al.*, 2000a). This inactivated NDP kinase is a useful tool for investigating the role of oligomeric structure, for example. It may be copurified with the endogenous NDP kinase of *E. coli*, leading to artifactual residual enzymatic activity. An easy way to overcome this complication is to mutate the active-site histidine, adding a histidine tag, and using a specific affinity column for purification (M.-L. Lacombe, personal communication, 1998).

The equilibrium constant of reaction (1a) (or the reverse of Eq. lb) has been found to be 0.15–0.5 using NDP kinases from several species (Garces and Cleland, 1969; Lascu *et al.*, 1983; Schaerlt *et al.*, 1998; Deville-Bonne *et al.*, 1996). The ΔG of hydrolysis of the phosphohistidine is more negative than the ΔG of hydrolysis of ATP. Unlike ATP, the phosphohistidine intermediate is kinetically unstable. These properties

make it a very potent phosphorylating agent, like its chemical counterpart phosphorylimidazole. Some of the unexplained secondary activities of NDP kinases are probably due to these properties (see below). Hydrolysis of the phosphoenzyme generates an NTPase secondary reaction. This reaction is different in nature from the NTPase reaction catalyzed by other phosphotransferases, such as hexokinase, in which water replaces the acceptor hydroxyl group of the second substrate. The NTPase activity of NDP kinase is much lower than that of the transfer reaction, less than 0.01% (Lascu *et al.*, 1983). The phosphorylated NDP kinase may be isolated. It has a half-life about 1h at room temperature, but is stable for long periods of time at −80°C. This intermediate resists the harsh conditions of electrospray mass spectrometry (Prinz *et al.*, 1999). The thiophosphorylated NDP kinase was found to have an even lower hydrolysis rate than the phosphorylated enzyme (Lasker *et al.*, 1999).

NDP kinases from different organisms, affected by mutation or using nucleotides modified in the γ position, may have different equilibrium constants for reaction (1a), as the enzyme interactions with the phosphoryl group may not be identical. A value of 5.0, ten times higher than that for wild-type enzyme (Schaertl *et al.*, 1999), has been reported for the equilibrium constant with the S 122P mutant of the human NDP kinase B. More NDP kinase may be phosphorylated at a given ATP/ADP ratio, but the catalytic properties of the enzyme should not be otherwise changed by modification of the equilibrium constant of the partial reactions. The apparent equilibrium constant also depends on the concentration of metal ions. It is higher in the presence of EDTA (nanomolar or lower concentrations of metal ions) than in the presence of millimolar concentrations of Mg^{2+} (Biondi *et al.*, 1998) because the MgADP complex is less stable the MgATP complex (Merouani, 1996).

The phosphorylated phosphohistidine intermediate may be isolated. As the equilibrium constant of reaction (1a) is less than 1, a large excess of NTP must be added to shift the equilibrium. Alternatively, a regenerating system (pyruvate kinase/phosphoenolpyruvate, for example) and a substoichiometric concentration of ATP may be used to phosphorylate the NDP kinase completely. One phosphate group is incorporated per NDP kinase subunit, under these conditions. This method may also be used to keep the enzyme phosphorylated during data collection over long periods of time, in [^{31}P]NMR experiments (Lecroisey *et al.*, 1995). The regenerating system has

been used to determine phosphorylation stoichiometry, using a spectrophotometric method (Lascu *et al.*, 1983). NTPase activity was measured in the same experiment.

The histidine has two non identical nitrogen atoms that may be phosphorylated. NDP kinases are phosphorylated at the N_δ position, as shown by X-ray crystallography (Moréra *et al.*, 1995) and [^{31}P]NMR analysis (Lecroisey *et al.*, 1995). N_δ phosphohistidine is more reactive than the N_ϵ isomer in model compounds, but N_ϵ is more stable. This result in phosphoryl group migration during sample preparation.

Phosphoramidate ($NH_2PO_3^{2-}$) has been found to be a poor substrate for NDP kinases. It tends to phosphorylate nitrogen nucleophiles rather than oxygen nucleophiles (Benkovic and Sampson, 1971). The incubation of *Drosophila* and *Dictyostelium* NDP kinase crystals with high concentrations of phosphoramidate made it possible, finally, to solve the crystal structure of the phosphorylated forms of the two enzymes. Only the active-site histidine was found to be phosphorylated (Moréra *et al.*, 1995). ATP is generated if NDP kinase is incubated with phosphoramidate and ADP (I. Lascu, unpublished data, 1994). An enzyme catalyzing the phosphorylation of nucleoside diphosphates by phosphoramidate was described several years ago in yeast (Dowler and Nakada, 1968). This enzyme may well be an NDP kinase because it has a ping-pong mechanism and was found not to be specific for the acceptor nucleotide.

THE NATURE OF THE RATE-LIMITING STEP IN THE NDP KINASE REACTION

For any discussion of enzyme mechanisms, we need to know which of the six steps in Scheme 1 are rate limiting for the catalytic cycle. This tells us which rate is measured in steady-state conditions. The correct design of the experiment is essential. Both of the half-reactions (1a) and (1b), contribute to the rate-limiting step in V_{max} conditions (saturating substrate concentrations). Conversely, each half-reaction may be analyzed separately in V_{max}/K_m conditions (low concentration of one substrate and saturating concentration of the other). The precise identification of the rate-limiting step is not easy. Partial information, concerning its nature (substrate binding/product dissociation or chemical transformation) may, however, be very useful.

The high k_{cat}/K_m measured for several NDP kinases, about 10^7 M^{-1}-s^{-1}, is similar to, but lower

than, typical values for diffusion-controlled enzymic reactions (10^8 M^{-1}-s^{-1}). We studied the effect of viscosity on k_{cat}/K_m for a series of nucleoside diphosphates. k_{cat}/K_m was proportional to the reciprocal of relative viscosity with TDP as substrate, and independent of relative viscosity for several TDP analogs modified in the 3′ position of the ribose. This strongly suggests (although it does not rigorously prove) that with the natural substrate, the rate-limiting step may be substrate binding or product dissociation. The chemical transformation (reaction 5) is "fast." With poor substrates, the chemical transformation (partial reaction 5 in scheme 1) becomes rate-limiting. Unfortunately, the rate of chemical transformation with the bound substrate (reaction 2 or 5 in Scheme 1) cannot be measured with good substrates. Any quantitative comparison between the kinetic parameters for a series of substrates, or between different mutants, should, therefore, be interpreted very carefully if the interaction of active-site residues in the transition state is to be discussed.

Studies of the effect of viscogens on k_{cat} showed that k_{cat} was proportional to the reciprocal of relative viscosity, with ATP and TDP as substrates. This suggests that both half-reactions are limited by diffusion (P. Gonin, unpublished results, 1998).

Figure 1 shows a proposed free-energy plot. This plot is qualitative because the free energy of some steps has not been or cannot be measured (for a discussion of free-energy plots, see Cleland and Northrop, 1999). With nucleoside diphosphates, which are poor substrates, the rate-limiting step of the catalytic cycle is the chemical transformation of bound substrate. This
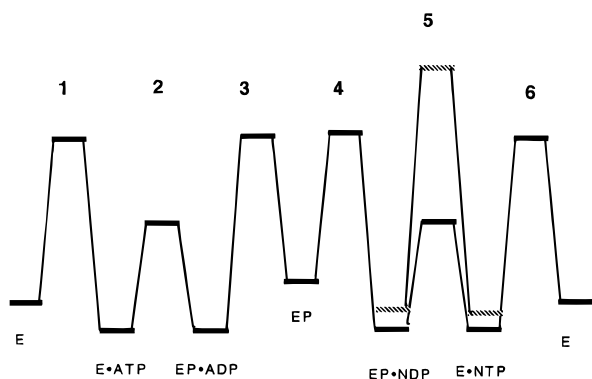

Fig. 1. Proposed free-energy profile for the reaction catalyzed by NDP kinases. The full line represents the reaction with good substrates, while the dashed line represents the profile with a nucleoside diphosphate, which is a poor substrate. The height of some barriers is hypothetical because the rate of the elementary steps is not known.

situation is much more informative for mechanistic purposes and could be used to estimate the effect of mutations and changes in physical conditions, such as pH. The reactions limited by diffusion have much lower activation energies than the reactions controlled by chemistry. With the *Dictyostelium* NDP kinase, we measured an activation energy of 5.5 ± 0.9 kcal/mol with ATP and TDP as substrates, whereas with ATP and AZT–DP, a poor substrate, the activation energy was 14 ± 2.9 kcal/mol (P. Gonin, unpublished results, 1998). The very different activation energies of two different steps in the catalytic cycle may responsible for the nonlinearity of the Arrhenius plots obtained with some NDP kinases (Agarwal and Parks, 1971).

A high k_{cat}, a k_{cat}/K_m controlled by diffusion, and a relatively high K_m for the substrates are the characteristics of the "perfect" enzyme, as defined by Alberty and Knowles (1976).

NATURE OF THE TRANSITION STATE OF THE NDP KINASE REACTION

The catalytic power of enzymes is due to the preferential stabilization of the transition state with respect to the ground state, the Michaelis complex. Because of its nature, the structure of the transition state cannot be studied directly. The E·ATP and Ep·ADP complexes are also refractory. Because of the duration of the crystallographic experiment, the secondary NTPase reaction takes place and the γ-phosphate is hydrolyzed. Cherfils and her colleagues (Xu *et al.*, 1997) used a different approach. They solved the crystal structures of the NDP kinase complexed with ADP·AlF₃, a transition state analog, and with ADP·BeF₃⁻, an ATP analog. Because of the unreactive nature of these analogs, a good image of the catalysis was obtained in two moments. Comparison of the two structures showed that there was no single stronger interaction with the transition state analog. The hydrogen bond between the ribose 3′-OH and the oxygen in ATP that acts as the bridge between the β- and γ-phosphates, becomes shorter. Figure 2 shows the active site of NDP kinase in the transition state, modeled on the structure of the enzyme–ADP–AlF₃ complex.

The structure of NDP kinase complexed with the transition state analog is of key importance as it can be used to determine the nature of the transition state. In principle, the distance between the attacking nucleophile and the leaving group (the histidine N_δ and the β-γ bridge oxygen of ATP) may vary in the transition
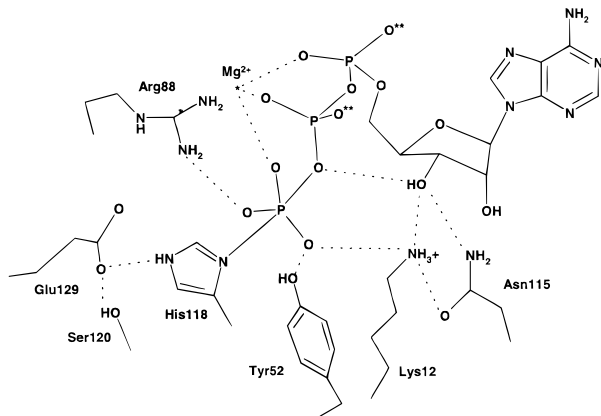
Fig. 2. Active site of bound NDP kinase in the transition state. The residue numbers are as for the human NDP kinases A and B. Adapted from Xu *et al.*, 1997b. The oxygen atoms marked by asterisks are replaced by sulfur in adenosine 5′-0-1-(thio)triphosphate S and adenosine 5′-0-2-(thio)triphosphate R, the diastereoisomers that are substrates for NDP kinase.

state, from the sum of the covalent radii in a completely associative mechanism, giving a distance of 3.4 Å, to the sum of the van der Waals radii for a completely dissociative mechanism (giving a distance of more than 5.0 Å; Knowles, 1980).The distance in the structure of the NDP kinase transition state analog was 4.8 Å, consistent with a largely *dissociative* character. A different result was obtained with the UMP kinase, for which the transition state was found to be *associative* (Schlichting and Reinstein, 1997). Understanding the nature of the mechanism is important because the charge distribution in the transition state is very different for the two mechanisms. Thus, some interactions may become critical if a charge develops on a particular atom in the transition state. In the dissociative mechanism, the oxygen bridging the β- and γ-phosphates becomes negatively charged. Recently, the importance for catalysis of stabilizing the developing charge of the leaving group in the transition state has been noted (Admiraal and Herschlag, 1995; Cepus *et al.*, 1998). For NDP kinases, the hydrogen bond with the ribose 3′-OH appears to be essential in catalysis. Replacement of the hydroxyl with other groups results in a large decrease in catalytic efficiency (Gonin *et al.*, 1999). This interaction defines "substrate-assisted catalysis." In addition to active-site residues, part of the substrate itself is involved in catalysis.

The involvement in catalysis of some of the active-site residues has been studied by site-directed mutagenesis. Lys12, Thr94, Arg88, Arg105, Tyr52, and Glu129 appear to be essential for catalysis. How-

ever, some of the mutations also affected enzyme stability (Tepper *et al.* 1994).

The k_{cat}/K_m for NDP displayed little variation with pH for values between 6 and 9 (k_{cat}/K_m is sensitive to protonation of the free enzyme and free substrates). His122, which cannot be protonated, should, therefore, have an exceptionally low pK_a. The decrease in activity obverved at pH values above 9 may be due to the deprotonation of Lys12 and Tyr52.

SECONDARY ACTIVITIES OF NDP KINASES

In addition to the NDP reaction, several secondary reactions have been described. One such reaction is the "autophosphorylation" of serine residues. As the active-site histidine was found to be necessary for this reaction, it was thought than the histidine of the NDP kinase was phosphorylated and that the phosphate group was then transferred to the serine. The crystal structure of the enzyme is not consistent with this. There is no serine close to the active-site histidine, except the serine in position +2 with respect to the histidine. This serine is hydrogen-bonded to a glutamate. Its phosphorylation would create considerable electrostatic repulsion. In addition, we found that the replacement of this serine with other amino acids greatly decreased stability of the enzyme (unpublished results). When measured, the molar ratio of serine phosphorylation was small, in some cases less than a few percentages (Bominaar *et al.*, 1994). It is possible that the phosphoryl group is transferred to the serine residue nonenzymically, in the *unfolded* state, in which the phosphohistidine and serine hydroxyl group may interact. As the phosphohistidine is a strong phosphorylating agent, the phosphoryl group may be easily transferred to water (the hydrolysis reaction). The serine hydroxyl has similar nucleophilic properties and pK_a; it can be phosphorylated with the same mechanism. The hydrolysis reaction is faster at higher temperature and at acidic pH. We found that benign sample preparation of phosphorylated NDP kinase for polyacrylamide gel electrophoresis resulted in most of the phosphate remaining attached to the histidine. If the sample was heated, or treated with acid, the proportion of "acid-stable" phosphorylation increased considerably (M. Erent, unpublished results, 1995). Therefore, the conditions used to discriminate serine phosphorylation from histidine phosphorylation promote phosphoryl migration from histidine to serine. All reports show

acid-resistant phosphorylation by electrophoresis under denaturing conditions. No study has demonstrated the existence of phosphoserine residues in the native NDP kinase. Experiments by gel filtration or electrophoresis under native conditions (in the presence of an excess of nucleoside diphosphate to scavenge the active-site phosphohistidine) and mass spectrometry, would help to solve this controversy.

NDP kinase has been found to phosphorylate the serine/threonine residues of other proteins. The reaction is stimulated by urea at nondenaturing concentrations. A nonenzymic reaction is likely because the transfer was stoichiometric rather than catalytic. Until a true enzymic reaction can be demonstrated, the significance of these findings is unclear.

Phosphoryl transfer from the phosphorylated NDP kinase to the histidine residues of other proteins has also been described. As the crystal structure is not consistent with there being physical contact beween the donor and acceptor to promote transfer, trace amounts of free nucleotides may mediate phosphate transfer. It has been found that nanomolar concentrations of ADP promotes phosphoryl transfer from the phosphorylated NDP kinase to CheA. This transfer does not occur without added ADP (Levit *et al.*, 1999). Phosphoryl transfer between phospho-HPr and HPr has also been shown to occur (Anderson and Waygood, 1993). In this protein, the active-site histidine is exposed on the protein surface, which is not the case for NDP kinases.

PHYSIOLOGICAL SIGNIFICANCE AND FURTHER DIRECTIONS

Since the review article of Parks and Agarwal (1973), substantial progress has been made toward understanding the catalytic mechanism of NDP kinases. However, their role in nucleotide metabolism is much less well understood. A few aspects of NDP kinase function at the cellular level will be described below. The preference of NDP kinases for some nucleotides has been demonstrated *in vitro*, but the *in vivo* relevance of this finding must be seen in the context of the cellular concentration of substrates and of the NDP kinase. In mammals, the concentration of ATP is much higher than that of other NTPs. ATP may be considered to be the only donor. In lower organisms, such as yeasts and *E. coli*, this is not the case. The difference in kinetic parameters with different nucleotides may be irrelevant if the NDP kinase reaction is

at equilibrium, as stated in textbooks. This is the case if the [NTP]/[NDP] ratio is equal to the [ATP]/[ADP] ratio. Unfortunately, cellular nucleotide concentration is seldom determined. The cellular turnover of the nucleotides is also important. The role of NDP kinase in UTP recycling may depend on glycogen being synthesized or not. NDP kinase activity differs greatly between organisms. For example, there is 10 times more NDP kinase activity per gram of tissue in *Drosophila* than in mammals. Specificity may, therefore, be more significant in mammals than in *Drosophila*. The discoveries of bacteria devoid of NDP kinases and that NDP kinase gene disruption has only minor effect in microorganisms raise new questions about the role of these enzymes.

Finally, the concept of "local synthesis" has been evoked but there has been no experimental demonstration of substrate channeling by complex formation. We await the results of these difficult, but important experiments with interest.

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