Nucleotide affinity for a stable phosphorylated intermediate of nucleoside diphosphate kinase

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Abstract

Nucleoside diphosphate (NDP) kinase is transiently phosphorylated on a histidine of the active site during the catalytic cycle. In the presence of a nucleotide acceptor, the phosphohistidine bond is unstable and the phosphate is transferred to the acceptor in less than 1 msec. We describe the synthesis of an analog of the phosphoenzyme intermediate with an inactive mutant of NDP kinase in which the catalytic histidine is replaced by a cysteine. In two sequential disulfide exchange reactions, a thiophosphate group reacts with the thiol function of the cysteine that had previously reacted with dithionitrobenzoate (DTNB). The thiophosphoenzyme presents a 400,000-fold increased stability in the presence of NDPs compared with the phosphoenzyme. The binding of NDP is studied at the steady state and presteady state. Data were analyzed according to a bimolecular association model. For the first time, the true equilibrium dissociation constants of NDP for the analog of the phosphoenzyme are determined in the absence of phosphotransfer, allowing a better understanding of the catalytic mechanism of the enzyme.

Keywords: Chemical modification; nucleoside diphosphate kinase; nm 23; phosphorylation

The transient phosphorylation of a variety of protein amino acids is a process frequently found in biological functions including cell cycle, signal transduction or catalysis. Alcohol residues such as Ser, Thr, and Tyr are the major targets of phosphorylation in proteins leading to modulation of enzymatic activities or to specific protein–protein interactions. The phosphorylation on a His residue is less frequent and phosphohistidine represents only 6% of the phosphorylated

proteins in prokaryotes and eukaryotes (Matthews 1995). Phosphohistidine residues are mainly observed in prokaryotes in so-called “two component systems” in which high energy phosphoryl groups are transferred from histidine to aspartate side chains (Stock et al. 1990). The His belonging to a “sensor domain” autophosphorylates, and the phosphate is transferred onto an Asp residue belonging to a “regulatory domain,” allowing it to trigger the relevant biological event. Phosphohistidines have also been observed in eukaryotic cells, mainly in signal transduction processes. Besides this regulatory role, phosphohistidine is also involved in the major group of phosphomonoesterases (the RHGXR group) including human prostatic and lysosomal phosphatases (Van Etten et al. 1991), in succinyl-CoA synthetase (Wolodko et al. 1994) and in phosphocarrier proteins (Herzberg 1992) and as a high energy intermediate during the phosphotransfer reaction catalyzed by nucleoside diphosphate (NDP) kinase (Garces and Cleland 1969).

NDP kinase catalyzes the phosphorylation of ribonucleosides and deoxyribonucleoside diphosphates into the corres-

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Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast-purification liquid chromatography; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; TNB[−], thionitrobenzoic ion; H122C, NDP kinase with His 122 replaced by Cys; F64W, NDP kinase with Phe 64 replaced by Trp.

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ponding triphosphates with adenosine triphosphate (ATP) as the major phosphate donor. The enzyme shows little specificity toward both the nucleobase and the ribose moiety and participates in the homeostasis of nucleoside triphosphate (NTP) pools as precursors for DNA and RNA synthesis (Parks and Agarwal 1973). Because it catalyzes the last step in the salvage pathway of nucleotide synthesis, NDP kinase is also involved in the phosphorylation of anti-human immunodeficiency virus (HIV) nucleoside analogs (Bourdais et al. 1996, Schneider et al. 1998b).

NDP kinases have been cloned from many organisms. They all show a high conservation in sequence and tridimensional structure. In prokaryotes, NDP kinases are made of four identical 17 kD subunits, whereas eukaryotic enzymes are hexamers. In humans, six isoforms have been identified. The gene *nm23-H1*, initially identified as a putative metastasis suppressor, encodes NDP kinase-A (Rosengard et al. 1989; Steeg et al. 1988; Wallet et al. 1990). NDP kinase-B binds to the NHE sequence of the *c-myc* proto-oncogene promoter and activates its transcription (Postel et al. 1993; Stahl et al. 1991). The *DR-nm23* gene product (also called NDP kinase-C) inhibits granulocyte differentiation (Venturelli et al. 1995), whereas Nm23-H4 is addressed to the mitochondrial compartment (Milon et al. 2000). Nm23-H5, specifically expressed in testis, is devoid of catalytic activity (Munier et al. 1998) as well as the *nm23-H6*, which is ubiquitously expressed (Mehus et al. 1999).

The crystal structures of several NDP kinases were solved as either unliganded proteins or as complexes with nucleoside diphosphates (Janin et al. 2000). Each subunit carries a single active site that can bind either an NDP or an NTP. At the surface of the active site, the nucleobase is sandwiched by Phe 64 with a hydrophobic contact to Val 116 on the opposite side of the base (Fig. 1). The ribose moiety is stabilized by an H-bond network. In addition, an intranucleotide H-bond also occurs between the 3'OH of the ribose and the O₇ of the β-phosphate. The phosphate chain, located deeper in the active site, is stabilized by two Arg residues. The γ-phosphate points toward a histidine residue that is transiently phosphorylated during catalysis.

The catalytic reaction of NDP kinases follows a bi-bi ping-pong mechanism as shown in Scheme 1:

where E ~ P is the phosphohistidine intermediate. The γ-phosphate of an NTP bound at the active site is transferred to the Nδ of the catalytic histidine during half-reaction (A) (Lecroisey et al. 1995). The resulting NDP leaves the active site and another NDP substrate binds at the phosphorylated active site for half-reaction (B) to occur. The

Fig. 1. Active site of *Dictyostelium* nucleoside diphosphate (NDP) kinase (1NDC.PDB).

kinetics of the nucleotide phosphorylation by NDP kinases have been studied both at the steady state and at the pre-steady state for most NDP kinases (Gonin et al. 1999; Schaertl et al. 1998; Schneider et al. 1998b). With natural nucleotides, the phosphotransfer in both directions proceeds in the ms time range. The structure of the phosphohistidine intermediate could, however, be successfully solved by X-ray crystallography with the enzymes from *Drosophila* and *Dictyostelium* on slow reaction with phosphoramidate (NH₂PO₃²⁻) (Morera et al. 1995). The comparison with NDP kinase bound to the transition state analog AlF₃-ADP showed few conformational changes between free and phosphorylated enzymes, except for a slight rotation of the catalytic histidine side chain, displacing the phosphate group that is bound by Tyr 56 (Xu et al. 1997). In the absence of nucleotides, the phosphohistidine bond is labile at acidic pH. For example, at 4°C and neutral pH, the phosphoenzyme is stable only for a few hours (Bominaar et al. 1994), and even at -20°C phosphohistidine was found to slowly hydrolyze (Lasker et al. 1999). We describe here the synthesis and characterization of a stable analog of the phosphorylated form of NDP kinase. We used a mutant of *Dictyostelium* NDP kinase in which the catalytic His was replaced by a Cys residue. This mutant which is totally devoid of catalytic activity led to the first crystallization experiments for NDP kinase for phasing (Dumas et al. 1992). We chemically modified Cys122 in H122C mutant NDP kinase. This resulted in a thiophosphate moiety mimicking the phosphohistidine that had a total loss of catalytic activity. The chemical stability of the phosphate linkage was found enhanced by several orders of magnitude compared with the native protein, even in the presence of nucleotides. This stable analog constitutes a useful model of the phosphory-

lated intermediate for measuring the affinities of NDP and NTP to the enzyme in the absence of phosphotransfer reaction.

Results

Synthesis of the phosphorylated analog of NDP kinase H122C-SPO₃²⁻

The chemical synthesis of H122C-SPO₃²⁻ was performed in two steps (Fig. 2) according to the procedure described for CheY (Silversmith and Bourret 1998). Each step released a TNB⁻ ion, which is detected by the increase in absorbance at 412 nm. In the first step, the mutant NDP kinase H122C reacted with dithionitrobenzoate (DTNB) in 4–5 min, and the product was desalted. The absorbance spectrum of the desalted H122C-TNB intermediate indicated a complete reaction with a ratio of $A_{326}/A_{280} = 0.55$ (not shown). The reaction of H122C-TNB with thiophosphate (HSPO₃²⁻) was complete within 100 min and led to a protein with no absorption peak at 326 nm, indicating the total removal of TNB⁻.

Isoelectric focusing (IEF) was used to estimate the amount of enzyme that reacted with the thiophosphate (Fig. 3). Wild-type NDP kinase displays a major band that corresponds to a pI of 7.90 (lane 2). This 'experimental' pI is more acidic than the predicted value from the amino acid sequence (pI = 8.92, EMBOSS, Sanger Center). Note that unmodified H122C mutant protein also displays a single band corresponding to a pI = 7.75, more acidic than the wild-type enzyme (lane 6). When the wild-type NDP kinase reacted with an excess of ATP and desalted on G-25 Sephadex column, the IEF pattern showed seven bands from pI 7.90 to 6.30 (lane 3). The most acidic isoform is attributed to the hexamer with the six subunits phosphorylated at the active site. The multiple bands are caused by the enzyme carrying 6 to 0 phosphate groups as a result of the instability of the phosphohistidine (Timmons et al. 1995). The reaction of H122C with DTNB leads to a major band at pI = 6.3 (lane 5), as expected from the presence of a negatively charged TNB⁻ group replacing the neutral cysteine. The reaction of H122C-TNB with thiophosphate leads to several bands (lane 4). The most acidic band (pI = 6.00), which is the most intense, corresponds to a hexamer in which the six

Fig. 3. Isoelectric focusing (IEF) of the protein intermediates in the chemical synthesis. Lane 1: IEF calibration kit (Pharmacia Biotech). Broad pI kit (pH 3–10). Lane 2: Wild-type NDP kinase. Lane 3: Phospho-intermediates of the wild-type NDP kinase. Wild-type NDP kinase was incubated with ATP for 30 min and separated using a G25 matrix. The seven bands correspond to different degrees of enzyme phosphorylation (from 0 to 6 phosphates per enzyme). Lane 4: Reaction of H122C-TB with HSPO₃²⁻ to >95% completion. The main band corresponds to the enzyme with a thiophosphate at each subunit. Lane 5: H122C-TNB. Lane 6: H122C.

subunits have released TNB⁻ ion and reacted with thiophosphate. Comparing the intensities of each band, we conclude that the predominant species (>95%) in our sample is the H122C protein with all its six subunits having reacted with HSPO₃²⁻ at the active site.

Fluorescence properties of H122C-SPO₃²⁻ NDP kinase and stability of the thiophosphate

The phosphorylation of NDP kinase is conveniently monitored by following the intrinsic fluorescence of the protein. In *Dictyostelium* NDP kinase, the single tryptophan (Trp 137) is close to the catalytic histidine in the protein core and its fluorescence emission is quenched (20%) on formation of the phosphohistidine. Conversely, when ADP is added to the purified phosphorylated intermediate, a 20% increase of the intrinsic fluorescence is observed on histidine dephosphorylation and ATP formation (Deville-Bonne et al. 1996).

Fig. 2. Chemical reaction leading to the modification of H122C mutant NDP kinase. The chemical changes occurring during the two sequential steps of the scheme are followed by measuring the (TNB⁻) at $\lambda = 412$ nm.

The fluorescence spectrum of the thiophosphate-H122C enzyme is shown in Fig. 4. When 20 mM of the strong reducing agent β -mercaptoethanol was added to 1 μ M H122C-SPO₃²⁻, a progressive increase of the intrinsic fluorescence was observed within 10 min, resulting from the release of the thiophosphate on disulfide bridge reduction. We conclude that the thiophosphate group on a H122C NDP kinase also quenched the tryptophan fluorescence as does the phosphate in the wild-type enzyme. This indicates that the Cys-thiophosphate moiety presents similar structural features found for the phospho-His derivative of the wild-type NDP kinase (Morera et al. 1995).

The chemical stability of the disulfide linkage in H122C-SPO₃²⁻ under normal storage conditions is of critical importance for biochemical studies. After several cycles of freezing at -20°C and thawing in 50 mM Tris at pH 7.5, the same fluorescence increase of the H122C-SPO₃²⁻ enzyme is still observed on reaction with β -mercaptoethanol (data not shown), indicating the high stability of the phosphorylated intermediate analog. However, when the H122C-SPO₃²⁻ (1 μ M) was incubated in T1 buffer at 20°C , the fluorescence slightly increased over time with a half-life of 35 min, that is, a rate constant of $k = 0.0003\text{ s}^{-1}$. When the thiophosphorylated enzyme sample was incubated in the presence of saturating nitrogen, the rate of the fluorescence variation was reduced by 20-fold (half-life $>11\text{ hr}$), indicating that the soluble O₂ is responsible for the oxidation of the disulfide bridge in the modified protein.

The stability of the H122C-SPO₃²⁻ NDP kinase was tested in the presence of ADP to check whether the thio-

phosphate could still react with an acceptor nucleotide. The transfer of phosphate from the NDP kinase H122C-thiophosphate to (¹⁴C)-ADP was compared with the rate of phosphorylation by the wild-type NDP kinase in the presence of GTP. The reaction products were separated by migration on thin layer chromatography (TLC) plates and counting by PhosphorImager (Molecular Dynamics). The incubation of H122C-SPO₃²⁻ (20 μ M) with (¹⁴C)-ADP (0.2 mM) during 30 min led to the formation of only a trace amount of (¹⁴C)-ATP, indicating a half-life of 35 min of the thiophosphoenzyme in the presence of ADP (data not shown). In the same conditions, the transfer of phosphate from the phosphorylated intermediate to ADP is instantaneous (in the msec range) and total (Schneider et al. 1998a). The rate of phosphotransfer from GTP to (¹⁴C)-ADP catalyzed by nonmutated NDP kinase amounts to 12 $\mu\text{M}/\text{min}/1\text{ nM}$ enzyme, as expected from a catalytic constant k_{cat} of 200 s^{-1} . In the presence of ADP, the comparison of the half-lives of the phosphoenzyme (5 msec) and of the thiophosphate-enzyme (35 min) indicates an enhancement of stability by a factor of 420,000; therefore in the presence of ADP, Cys-thiophosphate is then considerably more stable than the imidazol-phosphate. The rate of transfer of the thiophosphoenzyme in the presence of ADP amounted to the same low value previously found for the H122C-SPO₃²⁻ degradation in the absence of nitrogen bubbling. This rate process may correspond to conformational changes of the active site that activates the nucleotide by formation of an intranucleotide H-bond and allows the substrate-assisted catalysis.

Fig. 4. Fluorescence spectra of H122C-SPO₃²⁻ NDP kinase in the presence of β -mercaptoethanol. H122C-SPO₃²⁻ NDP kinase (1 μ M) was incubated in buffer T₁ saturated with nitrogen (●). 20 mM β -mercaptoethanol was added to the enzyme solution and the fluorescence emission intensity was recorded after 2 min (■), 10 min (◆), and 20 min (▼) on excitation at 295 nm (excitation slit: 2 nm).

NDP Affinity for H122C-SPO₃²⁻

The fluorescence of the H122C-SPO₃²⁻ NDP kinase (1 μ M) decreases by $\sim 50\%$ on addition of saturating amounts of guanosine diphosphate (GDP). We took advantage of this observation to determine the affinity of NDPs for the NDP kinase H122C-thiophosphate at the equilibrium. When GDP is added to NDP kinase H122C-SPO₃²⁻, a time-dependent quenching of the enzyme fluorescence is observed in the minute time scale with no observable lag (Fig. 5A). For all GDP concentrations tested, the intrinsic fluorescence follows a monoexponential progress, characterized by a first order rate constant k_{obs} . Note that the phenomenon observed here is totally different from the spontaneous reaction previously described. The binding of 0.2 mM GDP induces a decrease in fluorescence with a constant $k = 0.005\text{ s}^{-1}$, that is, 20 $\times$ faster than the previously described decomposition that produced an increase in fluorescence. The kinetics data are then analyzed according to the following model:

Fig. 5. Binding of guanosine diphosphate (GDP) to the phosphorylated intermediate H122C-SPO₃. (A) 1 μ M H122C-SPO₃ in buffer T₁ saturated with nitrogen at 20°C was mixed with 0.025–1.6 mM GDP (final concentration). The decrease in fluorescence was monitored with time at 340 nM. Each reaction was monitored during 2000 sec and the data are plotted on a 350-sec scale for more clarity. The solid lines represent the best fit of each curve to a monoexponential. (B) Concentration dependence on (GDP) binding of k_{obs} . The pseudo-first order rate constant for the interaction is linearly dependent on GDP concentration. (C) Concentration dependence on (GDP) binding of the fluorescence amplitude. The amplitude is hyperbolically dependent on (GDP) concentration. The solid line is the best fit of the data to an hyperbolic saturation curve.

where E-SP is the H122C-SPO₃²⁻ enzyme, k_{+1} the bimolecular association rate constant, and k_{-1} the dissociation rate constant, with K_D the true equilibrium dissociation constant between the phosphorylated enzyme and NDP the ratio

k_{-1}/k_{+1} . The first order rate constant of the reaction increases linearly with GDP concentration (Fig. 5B), as expected for a bimolecular reaction. The correlation coefficient measures the bimolecular association rate constant k_{+1} and is rather low with 18 $\text{M}^{-1}\text{s}^{-1}$. The intercept with the y-axis measures the dissociation rate constant k_{-1} . The amplitude signal increases as a saturation curve with a K_D of 40 μ M (Fig. 5C, Table 1), which agrees with the ratio $k_{-1}/k_{+1} = K_D$. Similar monoexponential decays were found when titrating the H122C-SPO₃²⁻ NDP kinase with ADP, dTDP, or CDP with a K_D of 100 μ M, 420 μ M, and 1.2 mM for ADP, dTDP, and CDP, respectively, in good agreement with the K_D values found from amplitude data (Table 1). When ATP was tested as a ligand of H122C-SPO₃²⁻ NDP kinase, a similar pattern was observed except that the affinity (>2 mM) could not be measured with accuracy (data not shown). These data show that the thiophosphoenzyme, a stable analog of the phosphoenzyme, binds the NDPs with an affinity at least 20-fold higher than the NTPs. Similar experiments were performed with the thiophosphate derivative of the double mutant H122C-F64W NDP kinase (see below) and GDP. Because the fluorescent signal variation was the same, all experiments were performed with the single mutant as described.

The oligomeric state of H122C-SPO₃ is not modified on ADP binding

The intrinsic fluorescence variation of H122C-SPO₃²⁻ NDP kinase on NDP binding could be attributed to several processes, such as a change in the enzyme conformation or a change in the oligomeric state. Because the enzyme is a hexamer, the dissociation of the stable phosphorylated intermediate into a lower form (tetramer or dimer) could occur on NDP binding. The elution profile from the Superdex 75 column indicates that the H122C mutant NDP kinase as well as the H122C-SPO₃²⁻ enzyme are homogeneous. They elute according to symmetric peaks at the same elution volume found for the wild-type enzyme (retention time = 8.8 min) (not shown). These data confirm that H122C and H122C-SPO₃²⁻ are hexameric proteins in the absence of

Table 1. Binding parameters of natural nucleotides to H122C-SPO₃²⁻ NDP kinase measured by fluorescence

	K_D^a (μ M)	k_{+1} ($\text{M}^{-1}\text{s}^{-1}$)	k_{-1} (s^{-1})	$K_D = k_{-1}/k_{+1}$ (μ M)
GDP	50	30	1.75×10^{-3}	58
ADP	100	20	1.60×10^{-3}	80
dTDP	420	4.5	1.90×10^{-3}	420
CDP	1200	1.5	2.80×10^{-3}	1800
ATP	>2000	n.d.	n.d.	n.d.

^a K_D values extracted from the amplitude data.

ligand. When ADP was added to H122C-SPO₃²⁻ NDP kinase, the elution profile was not modified, indicating that no dissociation occurred on ADP binding. We conclude that the change in enzyme fluorescence on NDP binding is attributable to a change in the active site conformation affecting the tryptophan fluorescence rather than to a change in the oligomeric structure of the modified enzymes.

Discussion

Unlike most of the kinases, NDP kinase has only one active site for both substrates, which bind each one in its turn. The NDP kinase is then transiently phosphorylated on the Nδ of histidine 122. During the catalytic cycle, the transition between the release of the first product (N₁DP) and the binding of the second substrate (N₂DP) by the phosphoenzyme remains unclear. The difficulties in studying histidine phosphorylation events are a result of the high energy state of the phosphate-imidazole bond that rapidly hydrolyzes. Several methods have been developed to increase the stability of the phosphohistidine residue and to preserve the overall structure of phosphohistidine, such as, for example, replacing the double bound oxygen of the phosphate with a sulfur atom. Because of its lower electronegativity, the sulfur atom stabilizes the imidazole N-P bond and decreases the hydrolysis sensitivity of the phosphohistidine (Lasker et al. 1999). Such a stabilization is not suitable for the study of the phosphorylated intermediate of NDP kinase, because NDP kinase is known to react with ATP-γS, although at a slower rate than with natural nucleotides (Goody et al. 1972; Schaertl et al. 1998). We have engineered a stable phosphohistidine analog as previously performed by Silversmith for CheY. The catalytic histidine of *Dictyostelium* NDP kinase was replaced by a cysteine residue by site-directed mutagenesis. For this, the *Dictyostelium* enzyme is of particular interest because it does not contain cysteine in its amino acid sequence. The reaction product H122C-SPO₃²⁻ was the predominant species, indicating the absence of secondary reactions (Silversmith and Bourret 1998). The stability of the phosphorylated intermediate analog was underlined by the very low phosphotransfer between ADP and H122C-SPO₃²⁻ NDP kinase. Moreover, no enzyme dissociation appeared on nucleoside diphosphate binding. Such a thiophosphoenzyme represents a relevant stable phosphohistidine analog.

The measure of the affinity of NDP for the H122C-SPO₃²⁻ NDP kinase was performed by use of the fluorescence of Trp 137, the single Trp in *Dictyostelium* NDP kinase, which is 50% decreased on NDP binding. As there is no dissociation of the hexameric substituted enzyme on NDP binding, we interpret our data as a change in the enzyme conformation that affects the fluorescence of Trp 137. The interaction of the H122C-SPO₃²⁻ enzyme and NDP is time dependent. The analysis according to a bimolecular

model shows a good correlation between K_D values obtained from the equilibrium data (amplitudes) and rate constants. Noticeable differences are shown for K_D values between nucleotides with the higher affinity obtained for GDP (K_D = 50 μM) (Table 1). The order of affinities GDP > ADP > dTDP > CDP agrees with the order of reactivity (Schaertl et al. 1998). Similar results were obtained with the thiophospho-derivative of the double mutant H122C.F64W NDP kinase that was designed to follow the binding of NDPs as described (Schneider et al. 1998b) (data not shown). Because both proteins led to the same fluorescence variation on NDP binding, most of the experiments were achieved with the single mutant as described. The fluorescence change can be attributed to a reorganization of the thiophosphate moiety that is presumably bound to Tyr 56 in the enzyme without NDP. In the X-ray structure of the phospho-intermediate of NDP kinase, the His 122 side chain was shown to undergo a rotation around the C_β-C_γ bond by approximately 30° (Morera et al. 1995). The interaction with NDP is likely to reorient the thiophosphate and, consequently, to modify Trp 137 fluorescence, as a result of the quenching properties of the disulfide bridge. The repulsion between phosphates of the NDP and the phosphate bound to the enzyme is likely to play a major role in the low efficiency of the interaction. Alternatively kinetic data could be analyzed according to a two-step reaction scheme, where the fast association of the enzyme with nucleotide is followed by a slow conformation change responsible for the fluorescent signal. Such a fit led to the same K_D values for nucleotide binding found with the previous model and to a second isomerization step with similar k₊ and k₋ constants, indicative of an equilibrium constant about 1 (not shown). As the second model did not give access to the association and dissociation rate constants of the nucleotide and both models led to the same K_D values for the nucleotides, it was reasonable to choose the simplest model for the analysis of our data.

Titration experiments with NDP and NTP have been previously performed with two mutant proteins, the F64W NDP kinase and the H122G.F64W inactive NDP kinase, respectively (Schneider et al. 1998b, 2000). The results concerning natural nucleotides are shown in Table 2. The introduction of a tryptophan in position 64 replacing Phe at the base-binding site provides a fluorescent signal to monitor the ligand binding. In this case, the equilibrium was reached instantaneously, allowing the measure of the affinity of NDP in the dead-end complex with NDP kinase. Despite the fact that the catalytic parameters of F64W NDP kinase were similar to those of the wild-type enzyme, the added Trp could contribute to enhance the apparent affinities as a result of increased stacking forces. The enzyme H122G.F64W NDP kinase is devoid of the catalytic His and the substrates' relative affinities could be determined. The free NDP kinase was shown to bind NTP with higher af-

Table 2. A comparison of the affinities of natural nucleotides for several mutants of *Dictyostelium* NDP kinase

K _D (μM)	H122G-F64W NDP kinase	F64W NDP kinase	H122C-SPO ₃ ²⁻ NDP kinase
GTP	0.15 ^a	—	—
ATP	0.20 ^a	—	>2000
dTTP	1.20 ^a	—	—
CTP	7 ^a	—	—
GDP	15 ^a	14 ^b	50
ADP	25 ^a	25 ^b	100
dTDP	75 ^a	100 ^b	420
CDP	210 ^a	200 ^b	1200

^a From Schneider et al. (2000). The mutant H122G.F64W NDP kinase is devoid of catalytic activity. It is a model to compare the binding of NTPs and NDPs with free NDP kinase.

^b From Schneider et al. (1998). The mutant H122G NDP kinase allows the study of the binding of NDPs to free active enzyme resulting in unproductive complexes. The similarity of the K_D values found for each NDP for these two mutants reinforces the validity of the model.

finities than NDP: the K_D for ATP and ADP were 0.2 μM and 20 μM, respectively, in a ratio of 100 (Table 2 and Schneider et al. 2000). The thiophospho-NDP kinase is shown here to bind ADP with a similar affinity, even slightly lower (K_D = 100 μM), whereas ATP binds with a very poor affinity (K_D = 2mM). These results indicate that the discrimination between nucleotides mainly results from NTP affinity changes. The relatively weak affinity of an NDP to the thiophosphoenzyme allows the easy release of N₁DP after phosphotransfer. The rather weak binding of N₂DP is compensated by the extremely high rate of phosphotransfer for the wild-type enzyme, resulting in an overall high efficiency of phosphotransfer. NTPs are the substrates with the best binding affinity. It thus appears that NDP kinase is present in the cell predominantly in a phosphorylated state. The equilibrium constant of NDP kinase with the nucleotide pools

$$K_{eq} = [\text{ADP}] \cdot [\text{EP}]/[\text{ATP}] \cdot [\text{E}] = 0.2$$

also agrees with the presence of a larger amount of phosphoenzyme. Because of its high concentration in cells (>1 μM), NDP kinase could serve as a reservoir of high energy bound phosphate.

Materials and methods

Materials

NDPs, dithiotreitol (DTT), and DTNB were from Sigma. Thiophosphate (HSPO₃) was purchased from Fluka. New England Nuclear provided (¹⁴C)-ADP (57 mCi/mmol).

Site-directed mutagenesis and enzyme purification

The mutation H122C in *Dictyostelium* NDP kinase was made by site-directed mutagenesis as described (Dumas et al. 1992). The

H122C mutant of *Dictyostelium* NDP kinase was overexpressed in *Escherichia coli* (XL1-Blue) by use of plasmid *pndk*, as described (Lacombe et al. 1990), with small modifications. The cell extract was loaded at pH 8.4 onto Q Sepharose FF, which retained only *E. coli* NDP kinase (Tepper et al. 1994), and the flow-through was adsorbed on Orange-A (Amicon) at pH 7.5. After washing with Tris buffer, the enzyme was eluted by a gradient of NaCl (0–1.8 M) in 50 mM Tris-HCl at pH 7.5. After dialysis, the protein was concentrated with an Amicon ultrafiltration cell, equilibrated in 50 mM Tris-HCl at pH 7.5 and stored at –20°C. The H122C mutant enzyme was purified to homogeneity as judged by SDS-PAGE.

Synthesis of H122C-SPO₃²⁻

Concentrated aliquots of purified H122C enzyme were reacted with 10 mM DTT for 10 min at room temperature to completely reduce the single cysteine thiol (Silversmith and Bourret 1998). The protein was then desalted on a G-25 Sephadex Column (10 × 25 mm) equilibrated in 100 mM Tris at pH 7.5, ethylenediaminetetraacetic acid (EDTA) 1 mM. Protein-containing fractions were pooled (~1 mL, 40 μM) and reacted with a 10 M excess of DTNB (10 mM stock solution prepared immediately before use). The time-course of the reaction was monitored by following the increase in absorbance at 412 nm. The change in absorbance was corrected for an absorbance increase caused by a spontaneous DTNB hydrolysis in the same buffer. The final absorbance at 412 nm was used to calculate the TNB[–] ion concentration using an extinction coefficient $\epsilon_{412\text{nm}}^{\text{TNB}^-} = 13,600 \text{ M}^{-1}\text{cm}^{-1}$. When the reaction was completed (~4 min), the modified protein (H122C-TNB) was separated from DTNB/TNB[–] by gel filtration on G-25 Sephadex (column size 10 × 25 mm) in 100 mM Tris at pH 7.5, EDTA 1 mM. The H122C-TNB pool (~2 mL, 35 μM) was then reacted with a 15 M excess of freshly prepared HSPO₃^{2–}. Again, the time course of the reaction was followed by monitoring the increase in absorbance at 412 nm as result of TNB[–] release. The reaction was completed in ~100 min and the sample was desalted using G-25 Sephadex (10 × 25 mm) equilibrated with 50 mM Tris at pH 7.5. Protein concentration was determined using an absorbance coefficient of A₂₈₀ = 0.59 for a 1 mg/mL solution according to Gill equation (Gill and Von Hippel 1989). Enzyme concentration was expressed as subunit concentration using MW = 17,000.

Radioactive assay of phosphotransfer by NDP kinase on thiophosphate analogs

To assess the stability of the phosphorylated intermediate, the H122C-SPO₃^{2–} was tested as a possible donor of phosphate using (¹⁴C)-ADP as an acceptor. The H122C-SPO₃^{2–} (20 μM) was incubated with a constant amount of (¹⁴C)-ADP (0.2 mM) during 30 min. After separation of the nucleotides by TLC on PEI cellulose (Macherey-Nagel, Germany), their radioactivity was quantified using a PhosphorImager (Molecular Dynamics).

IEF

IEF was performed using IEF phast gel (Amersham Biosciences) with a pH range from 3 to 9. All protein samples were in 50 mM Tris at pH 7.5. Samples (5 μg) were electrophoresed horizontally for ~30 min at 2000 V. The gel was first fixed with 20% trichloroacetic acid for 5 min at 20°C, then washed with 30% methanol and 10% acetic acid for 2 min at 20°C and stained with 0.04% Coomassie R-250 in 27% isopropanol and 10% acetic acid for 10 min

at 50°C. The IEF gel was destained with the same solvent used for the wash step for 10 min at 50°C. The pIs of intermediates that were generated during H122C-SPO₃²⁻ synthesis were estimated assuming a linear pH gradient from 3 to 9. An IEF calibration kit containing pI markers (pH 3–10) was also used for accurate estimation of pIs of H122C and derivatives.

Fluorescence measurements

The affinity of NDPs for the phosphorylated intermediate analog of H122C-SPO₃²⁻ NDP kinase was measured by following the variation of the intrinsic enzyme fluorescence on NDP binding. All fluorescence measurements were performed at 20°C with continuous stirring in buffer T₁ (50 mM Tris-HCl at pH 7.5, containing 5 mM MgCl₂ and 75 mM KCl) saturated by nitrogen with a Photon Technology International spectrofluorometer Quantamaster. The kinetic of NDP binding to H122C-SPO₃²⁻ protein was followed on nucleotide addition to the phosphorylated intermediate (1 μM) in buffer T₁ in a four optical windows Hellma cell (1 mL volume, 1 cm optical path). The mixing time was less than 15 sec. The Trp fluorescence of H122C-SPO₃²⁻ was monitored for up to 17 min at 340 nM with excitation at 295 nm for ADP or 304 nm for other nucleotides (2 nm excitation slit and 4 nm emission slit) to avoid light absorption by the nucleotides. Experimental curves followed monoexponential progress. The amplitude of the fluorescence signal was plotted as a function of NDP concentration (titration curve). The pseudo-first order rate constant (k_{obs}) was also determined as a linear function of NDP concentration.

Oligomeric state of H122C-SPO₃²⁻ protein

The oligomeric state of H122C-SPO₃²⁻ free or in complex with ADP was performed by fast-purification liquid chromatography (FPLC) gel filtration with a Superdex 75 column (Amersham Biosciences) equilibrated in buffer T₂ (50 mM Tris-HCl at pH 7.5 containing 5 mM MgCl₂ and 200 mM KCl) and developed at a flow rate of 0.4 mL/min. In some experiments, the elution buffer was buffer T₂ containing 200 μM ADP. The protein elution was followed at 280 nM and 295 nM.

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