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NUCLEOSIDEDIPHOSPHATE KINASE OF *ESCHERICHIA COLI*, A PERIPLASMIC ENZYME

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Summary

The ATP-ADP exchange activity previously described in a membrane fraction of *Escherichia coli* appeared after a cold osmotic shock according to Neu and Heppel ((1965) J. Biol. Chem. 240, 3685—3692) in the shock fluid. Membranes derived from shocked cells had no activity.

The enzyme responsible for this activity has been purified 125-fold and catalyzed the transfer of a phosphoryl radical from ribonucleosidetriphosphates (NTPs) to ribonucleosidediphosphates (NDPs); this is, therefore, a non-specific nucleosidediphosphate kinase (ATP:nucleosidediphosphate phosphotransferase, EC 2.7.4.6). The activity required the presence of a divalent cation, Mg^{2+}, Mn^{2+} or Ca^{2+} at a unity mol/mol ratio of nucleotide for maximal activation.

The enzyme exhibited simple saturation kinetics with respect to the phosphate donor but inhibition by excess substrate was observed upon increasing phosphate acceptor. The kinetics of the reaction indicated an ordered bi-molecular ping-pong reaction mechanism.

Differential heat sensitivity of the enzyme whether it is heated alone with ATP, ADP or Mg^{2+} opens possibilities to study different enzyme-substrate complexes.

Introduction

In a previous report on the membrane ATPase of *Escherichia coli*, we have described an activity in the same preparation, the ATP-ADP exchange [1]. The question was to know if the ATPase activity and the ATP-ADP exchange activ-

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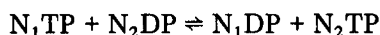
ity were due to the same enzyme. In favor of this hypothesis, we have observed that the inhibition of ATPase activity upon addition of ADP, excess Mg^{2+}, P_i or azide was accompanied by the stimulation of ATP-ADP exchange activity. We interpreted this as the synthesis of a phospho-enzyme as the first partial reaction of ATPase, which could not be followed by hydrolysis, the second partial reaction, due to the presence of the above inhibitors. Like the ATP-ADP exchange reaction catalyzed by $(Na^+ + K^+)$-ATPase in the presence of *N*-ethylmaleimide [2]. The two activities were released from the membranes by the same treatment, namely a 20-fold dilution, into a Mg^{2+}-free buffer of low ionic strength [3]. However, these enzymes were separated by chromatography on Sephadex G-200. Moreover, we later found that membrane particles obtained from bacteria submitted to an osmotic shock according to Neu and Heppel [4] had no ATP-ADP exchange activity while ATPase activity remained normal. The exchange activity was recovered in the osmotic shock fluid.

The activity of ATP-ADP exchange is catalyzed by a nucleosidediphosphate kinase (ATP:nucleosidediphosphate phosphotransferase EC 2.7.4.6) which transfers the terminal phosphate of a nucleosidetriphosphate (NTP) to a nucleosidediphosphate (NDP), according to the reaction:



$$N_1TP + N_2DP \rightleftharpoons N_1DP + N_2TP$$

ATP-ADP exchange is not the only reaction catalyzed by this enzyme which exhibits little specificity with regard to the NTP and NDP substrates.

In this communication we report a detailed study of the kinetic behavior of the purified NDP kinase prepared by osmotic shock of *E. coli*. The data presented suggest a catalytic mechanism of the ordered bi-molecular ping-ping type [5]. Ion dependence, molecular weight, heat sensitivity of the enzyme are also described.

Materials and Methods

Bacterials strain. E. coli strain K108, a galactokinase-deficient strain was used in these experiments.

Bacteria were grown on medium 63 [6] supplemented with glycerol as described previously [1].

Osmotic shock. The modification [7] of the method published by Neu and Heppel [4] was used.

Bacteria were resuspended and washed with 10 mM Tris-HCl buffer (pH 7.3)/30 mM NaCl at 0°C. The pellet was resuspended in 10 mM Tris-HCl buffer (pH 7.3)/0.5 M sucrose/0.1 mM EDTA. The suspension was gently stirred for 15 min at room temperature and was centrifuged. The pellet was quickly resuspended in ice-cold 0.5 mM $MgCl_2$ and, after 5 min stirring in the cold, the cells were removed by centrifugation. The shock fluid was concentrated by ultrafiltration on hollow fiber filter (Amicon HIP 100). The shock procedure was controlled by comparing the rate of uptake of [^{14}C]galactose [8] of shocked and unshocked cell suspensions.

Preparation of membranes. The membranes were obtained as described previously [1]. In brief high pressure extrusion at 20 000 lb/inch^2 by a Sorvall Ribi cell fractionator was followed by differential centrifugation. Particles sedi-

menting between 30 000 × *g* and 150 000 × *g* were collected and stored in liquid N_2.

Enzyme assays. The activity was determined by measuring either the amount of [^{14}C]ADP formed from [^{14}C]ATP, or [^{14}C]ATP formed from [^{14}C]ADP by radiochromatographic method as described previously [1]. The reaction mixture contained in 50 mM triethanolamine buffer (pH 7.5), 3 mM ATP, 1 mM ADP, 14 mM Mg^{2+} and enzyme solution. Samples were spotted onto paper and developed in isobutyric acid/1 M amonia/1 mM EDTA (100 : 60 : 1.6, v/v). The product radioactivity was plotted as a function of time and the slope was taken as the initial velocity, when the product was less than 25% of the final isotopic equilibrium. When reactions progressed at 25—50%, the initial velocity was calculated from a semilog plot of the extent of reaction vs. time. For enzyme solutions which contained ATPase activity, 2 mM NaN_3 was added.

ATPase activity was measured as described previously [1].

Purification of NDP kinase. All experiments were carried out at 4°C.

Gel filtration on Sephadex G-100. The concentrated shock fluid was applied to a Sephadex G-100 column (80 × 2.5 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.6) and eluted with the same buffer. Protein concentration was followed by recording ultraviolet absorption with an ISCO model UA 4 absorbance monitor. The elution pattern is presented in Fig. 1. 15-ml fractions were assayed and those containing significant NDP kinase activity were pooled.

DEAE-cellulose chromatography. The Sephadex G-100 enzyme pool was applied to a DEAE-cellulose column (DE-52, Whatman; 10 × 1.6 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.2). The column was eluted first with 100 ml equilibrating buffer followed by 400 ml of a linear gradient of 10—50 mM Tris-HCl (pH 7.6) from which 8-ml fractions were collected active fractions were eluted at 200—300 mM and pooled (Fig. 2). This fraction was concentrated by lyophilization, resuspended in 4 ml of 10 mM Tris-HCl buffer (pH 7.6) and dialyzed overnight against the same buffer.

Gel filtration on Indubiose (acrylamide-agarose) AcA3-4. The dialyzed enzyme solution was applied to an Indubiose AcA3-4 column (26 × 2.5 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.6) and eluted with the same buffer. The elution volume for NDP kinase was 92 ml. This fraction was

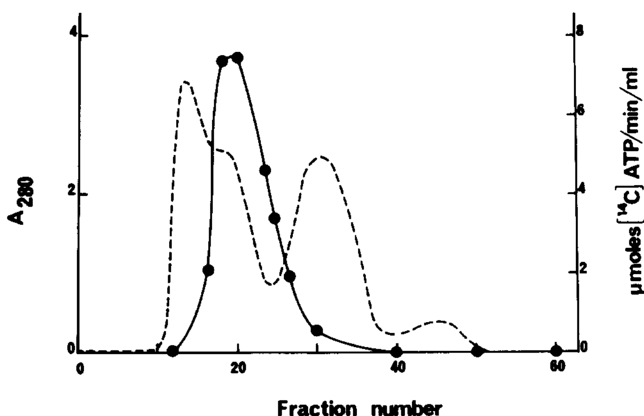


Fig. 1. Gel filtration on Sephadex G-100. - - - - - -, A_{280nm}; ●———●, NDP kinase activity.

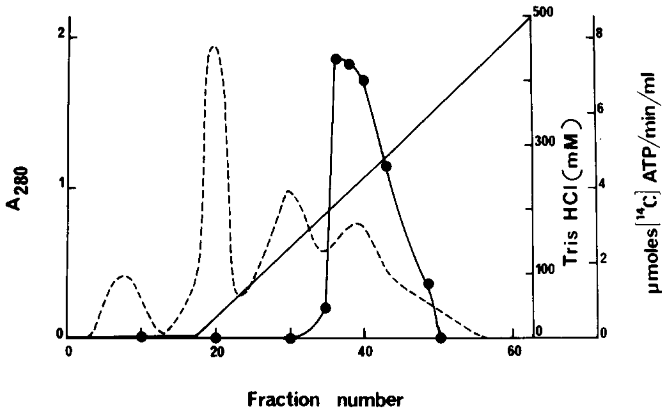


Fig. 2. Chromatography on DEAE-cellulose. A linear gradient of 200 ml 10 mM Tris-HCl buffer (pH 7.6) and 200 ml 500 mM Tris-HCl buffer (pH 7.6) was used to elute proteins; - - - - - -, A_{280nm}; •———•, NDP kinase activity.

lyophilized. The purified enzyme could be stored at —20°C for as long as 1 year with no significant loss in activity.

Sucrose density gradient centrifugation. The sedimentation coefficient was determined by sucrose density gradient centrifugation according to the method of Martin and Ames [9].

The enzyme solution was applied to 5 ml of 5—20% sucrose gradient in 1 mM Tris-HCl buffer (pH 7.6) and run at 4°C in the SW50L rotor at 37 000 rev./min for 18 h.

Results

Effect of treatment of Neu and Heppel on the localization of NDP kinase. As shown in Table I, when the treatment of Neu and Heppel [4] was omitted, the membrane particles contained the two activities ATPase and NDP kinase. After pretreatment the final preparation of membranes was virtually free of NDP

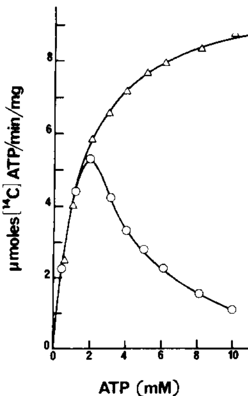


Fig. 3. Effect of ATP concentration at two different concentration of Mg^{2+}. The experiments were carried out with 1 mM [^{14}C]ADP. ○———○, 1.5 mM Mg^{2+}; △———△, 14 mM Mg^{2+}.

TABLE I

DISTRIBUTION OF ATPase AND NDP KINASE ACTIVITIES DURING FRACTIONATION

Specific activities are expressed as μmol product/min per mg protein, and *total* activities as μmol product/min.

	Protein (mg)	ATPase			NDP kinase		
		Specific activity	Total activity	Yield (%)	Specific activity	Total activity	Yield (%)
Broken bacteria	4000	0.157	628	100	0.20	800	100
Pellet (30 000 × *g*—160 000 × *g*	1140	0.470	535	83	0.52	592	74
Supernatant 160 000 × *g*	1840	0.050	92	14	0.06	110	13
Fluid from cold osmotic shock	510	0.010	—	—	1.60	816	81
Broken bacteria pretreated according to Neu and Heppel	3600	0.190	684	100	0.05	180	18
Pellet (30 000 × *g*—160 000 × *g*	1280	0.480	614	89	0.04	62	6
Supernatant 160 000 × *g*	1000	0.070	70	10	0.06	60	6

TABLE II

PURIFICATION OF NDP KINASE

The activities were measured at pH 7.5 with 3 mM ATP/1 mM [^{14}C]ADP/14 mM Mg^{2+}.

	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (μmol [^{14}C]-ATP/min per mg protein)	Yield (%)	Purification (-fold)
Shock fluid	2000	0.255	510	1.6	100	1
Sephadex G-100	110	1.0	110	5.1	68	3.18
DEAE-cellulose eluate concentrated	4	8.55	34.2	10	42	6.25
Indubiose AcA3-4	49	0.22	10.6	25	30	15.62

kinase activity. This activity was present in the shock fluid and ATPase was localized on the membrane.

Purification of NDP kinase. The scheme of purification of NDP kinase from shock fluid is summarized in Table II. A 15-fold purification was achieved with a final yield of 30%. This is a 125-fold enrichment in specific activity when referred to a crude extract (see broken bacteria, line 1 of Table I).

NDP kinase had a broad pH optimum, essentially equally active between pH 7 and 9 and retaining about 2/3 optimal activity at pH 6 and 9.5. The experiments described below were carried out at pH 7.5.

Effect of divalent cations. Previous studies on the ATP-ADP exchange reaction catalyzed by membranes showed that the enzyme has a requirement for Mg^{2+}.

The experiments of Fig. 3 were carried out in the presence of 1 mM ADP and 1.5 mM Mg^{2+} with increasing concentrations of ATP. When the ATP concentration exceeded 2 mM, there was progressive inhibition of the reaction. Under these conditions, Mg^{2+} concentration became insufficient. The same figure shows that, with a high concentration of Mg^{2+} (14 mM), ATP concentration dependence curves followed normal saturation kinetics and there was no inhibtion by excess ATP.

All enzyme activity measurements reported below were carried out with a ratio of $Mg^{2+}/(N_1TP + N_2DP) \geqslant 1$.

In order to determine cation requirement of the enzyme, reaction mixtures containing 3 mM ATP and 1 mM ADP were supplemented with increasing concentrations of $MgCl_2$. The activity optimum was reached at 4 mM of Mg^{2+}, i.e. when the ratio of Mg^{2+} to (ATP + ADP) was one. The optimal 1/1 ratio between Mg^{2+} and (ATP + ADP) was confirmed at different concentrations of ATP.

From this experiment, it can be concluded that the enzyme accepts as substrates MgATP and MgADP.

The enzyme was also activated by two other cations used: Mn^{2+} and Ca^{2+}. The Mn^{2+} had the same effectiveness as Mg^{2+} and at 4 mM Ca^{2+} was about 30% less effective than Mg^{2+}. All three cations gave half maximal activation at about 1 mM.

Substrate specificity of NDP kinase. The substrate specificity of the enzyme

TABLE III

ACTIVITY OF NDP KINASE ON NTPs AND NDPs

The reaction mixture contained: 3 mM NTP, 1 mM NDP, 14 mM Mg^{2+}, 50 mM triethanolamine buffer, (pH 7.5) and 4 μg enzyme from the concentrated DEAE-cellulose eluate.

Nucleoside triphosphate	Nucleoside diphosphate	μmol [^{14}C]ADP/min	μmol [^{14}C]ATP/min	Activity (%)
[^{14}C]ATP	ADP	33.3		100
[^{14}C]ATP	UDP	33.3		100
[^{14}C]ATP	CDP	27.8		83.5
[^{14}C]ATP	GDP	24.2		72
ATP	[^{14}C]ADP		36	100
UTP	[^{14}C]ADP		35.4	78
CTP	[^{14}C]ADP		24.8	69
GTP	[^{14}C]ADP		22	61

was studied using various NDPs in the presence of [^{14}C]ATP, and a variety of NTPs in the presence of [^{14}C]ADP (Table III).

All the NDPs tested could serve as acceptors of P_i and all the NTPs could serve as donors of P_i.

The rate of ATP-UDP exchange was the same as the rate of ATP-ADP exchange, other combinations gave reaction rates between 50 and 100% of the former.

Like many NDP kinases isolated from other sources, the enzyme of *E. coli* was found to be non-specific with respect to substrates [10,11].

A variety of compounds were examined as possible substrates or inhibitors of the enzyme. AMP, acetate, ribose, adenosine, NADP, pyrophosphate or phospho*enol*pyruvate, could not play the role of phosphate acceptor with ATP as the donor, and ADP, AMP, PP_i or phospho*enol*pyruvate could not play the role of phosphate donor with ADP as the acceptor.

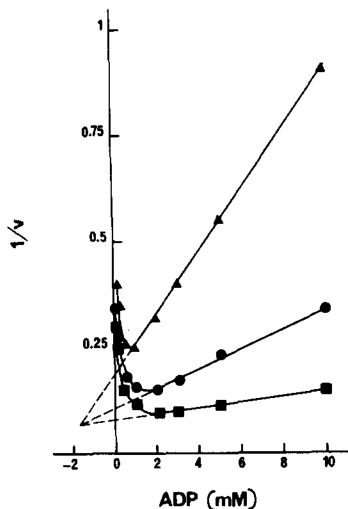
Among these substances only PP_i caused a significant inhibition of the enzyme (50% at 20 mM, 100% at 50 mM). This might be ascribed to the chelation of Mg^{2+} by PP_i.

Kinetic studies. Fig. 4 shows the velocity of NDP kinase with ADP as the variable substrate at different fixed concentrations of ATP.

We showed an inhibition of NDP kinase reaction by an excess of ADP. This inhibition could be released by increasing the concentration of ATP, but not by increasing the concentration of Mg^{2+}. Fig. 4 shows that ADP acts as a competitive inhibitor of ATP (K_i = 1.8 mM). When GDP was used as phosphate acceptor instead of ADP, GDP also gave competitive substrate inhibition at high concentration (K_i = 0.8 mM).

At non-inhibitory concentration of ADP, the double reciprocal plots of reaction rate vs. ATP concentration yield a family of parallel lines (Fig. 5). From the data a Michaelis constant for ATP of 1.55 mM and for ADP of 0.25 mM can be calculated.

An identical pattern was obtained for the reaction involving ATP and GDP, when GDP was non-inhibitory. The constants derived from these experiments are given in Table IV, line 4.



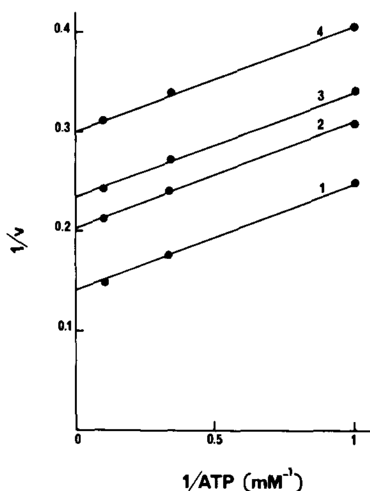


Fig. 4. Inhibition by excess ADP of NDP kinase activity. Plot of the reciprocal of the initial velocity (μmol [^{14}C]ADP/min per mg) versus ADP concentration. The experiments were carried out with 21 mM Mg^{2+}: ▲——▲, 1 mM ATP; ●——●, 3 mM; ATP; ■——■, 10 mM ATP.

Fig. 5. Effect of ADP on the NDP kinase activity. Plot of the reciprocal of the initial velocity (μmol [^{14}C]ADP/min per mg) against the reciprocal of the ATP concentration at different ADP concentrations. 1, 0.5 mM ADP; 2, 0.2 mM ADP; 3, 0.15 mM ADP; 4, 0.1 mM. ADP.

Inhibition between NDPs. In a reaction where ADP was used as acceptor and GTP as donor, the addition of GDP was inhibitory for ATP production. At different concentrations of ADP, when GDP was varied, it appeared as a competitive inhibitor of ADP (K_i = 0.37 mM). (Table IV, line 5).

Inhibition between NTPs. GTP acts as a competitive inhibitor of [^{14}C]ATP (K_i = 2.25 mM) for [^{14}C]ADP production (Table IV, line 6).

Inhibiton between GTP and GDP. When GDP was the variable substrate, in the concentration range of 1 mM and below, and [^{14}C]ATP the fixed substrate at non-saturating concentration (1.5 mM), at various concentrations of GTP, a pattern of non-competitive inhibition was observed between GTP and GDP. We

TABLE IV

KINETIC CONSTANTS OF NDP KINASE WITH VARIOUS NUCLEOTIDES

Values were obtained from appropriate plots of data. K_i was determined from secondary plots of 1/([^{14}C]ADP formed from [^{14}C]ATP vs. (GDP) at various GTP concns. (a) slope vs. GTP concn., (b) intercept vs. GTP concn.

Reaction	Ligand	K_m (mM)	K_i (mM)
[^{14}C]ATP + ADP → [^{14}C]ADP + ATP	ATP	1.55	—
	ADP	0.25	1.8
[^{14}C]ATP + GDP → [^{14}C]ADP + GTP	ATP	1.43	—
	GDP	0.47	0.8
GTP + [^{14}C]ADP → GDP + [^{14}C]ATP	GDP against ADP		0.37
[^{14}C]ATP + GDP → [^{14}C]ADP + GTP	GTP against ATP		2.25
	GTP against GDP		2.5 (a) 4.75 (b)

observed that at high level of ATP, GTP had no effect on the rate of the reaction (Table IV, line 7).

Determination of the molecular weight of NDP kinase. The sedimentation coefficient and the molecular weight of the enzyme were estimated by sucrose density gradient centrifugation. β-Galactosidase of *E. coli* (16 S) catalase (11.1 S) and human hemoglobin (4.4 S) were used as standards for molecular weight. The sediementation constant of NDP kinase was found to be 5.9 S. From the data, using the equation of Martin and Ames [9], a molecular weight of 110 000 was determined.

The molecular weight of the enzyme was also estimated from its elution volume on Indubiose AcA3-4 [12]. The molecular weight was estimated to be about 115 000. This value is in good agreement with the value determined by sucrose gradient centrifugation.

Effect of temperature on enzyme activity. Samples of the enzyme in 10 mM Tris-HCl (pH 7.6) were heated to different temperatures for 1 min, then immediately cooled in an ice bath and subsequently assayed for enzyme activity. Half-inactivation was reached under these conditions at 51° C. Inactivation was complete at 60° C.

Fig. 6 shows the kinetics of inactivation of the enzyme at 51° C in the presence of ATP, ADP, ATP-Mg^{2+} and ADP + Mg^{2+}.

It can be seen that ATP protected the enzyme against inactivation by heat raising its half-life from 2 to 4 min, whereas ADP, ADP-Mg^{2+} sensitized the enzyme, decreasing its half-life to 0.5 min while ATP-Mg^{2+} or Mg^{2+} alone decreased the $t_{1/2}$ to 1 min.

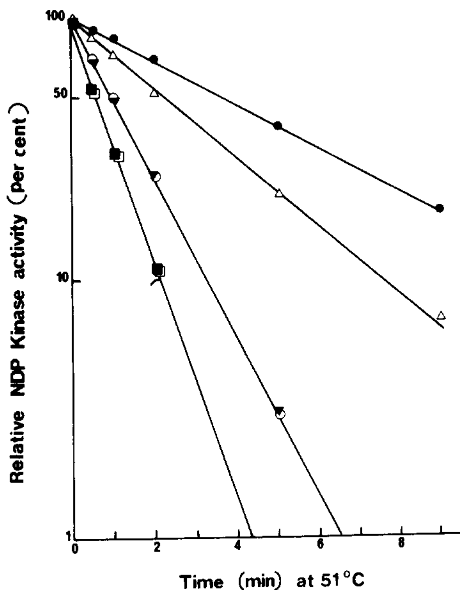


Fig. 6. Time course of heat inactivation of NDP kinase at 51° C. Concentrated enzyme with different addition was heated at 51° C. Small volume samples were diluted at intervals in large volume of incubation buffer. Additions: △——△, control; ●——●, 10 mM ATP; ○——○, 10 mM ATP + 10 mM Mg^{2+}; □——□, 5 mM ADP; ▼——▼, 10 mM Mg^{2+}; ■——■, 10 mM ADP + 10 mM Mg^{2+}.

Discussion

NDP kinases have been described in many species and in many tissues [10,11]. Besides, enzymes located in the cytosol of higher animals [13], the mitochondrial enzyme has attracted much attention [14,15]. Part of this has been found in the outer mitochrondrial membrane and part of it in the intermembrane space of mitochondria [16]. In *E. coli*, DNA polymerase I has been described as having a NDP kinase activity [17]. Unlike DNA polymerase, the enzyme here described is relased from *E. coli* following a cold hypo-osmotic schock according to Neu and Heppel [4] is therefore presumably located in the space between outer and inner membranes of the bacteria. Since it was first described in a particulate preparation, it can be visualized as a protein loosely bound to the outer surface of the inner membranes of *E. coli*, or to the inner face of the outer membrane. Since any kinase with a donor specificity not restricted to ATP could perform the reactions here ascribed to NDP kinase by a reversal of its first partial reaction, the exact role of the enzyme here described can be questioned. None of the potential phosphate acceptors tried was recognized by the enzyme while its affinity for NDP acceptors appeared stronger than its affinity for NTP donors. This is in favor of a real NDP kinase. Whether this is so or not, the periplasmic location of the enzyme, although reminiscent of the situation in mitochondria, poses particularly puzzling questions. What could be the physiological role of a periplasmic kinase with free living organisms having NTPs only in their cytoplasm as far as we know. Nevertheless, an antibiotic, desdanine or cyclamidomycine appears to be a specific inactivator of bacterial NDP kinase [18], emphasizing thereby its physiological importance.

Most NDP kinases described up to now have molecular weights ranging between 80 000 and 115 000 [19—21]. All those which have been carefully studied required the presence of divalent cations and all of them exhibit an activity pattern consistent with bimolecular ping-pong reaction mechanisms, where one product is released before the second substrate combines with the enzyme [5,10,11,14,19,22,23]. Nevertheless ADP can also combine to the ATP site of the enzyme presumably of its non-phosphorylated form as indicated by the competitive inhibition exerted by excess ADP against ATP and by the increased heat sensitivity of the enzyme in the presence of ADP.

Our finding that the heat stability of NDP kinase can be changed to more resistance by complex formation with the phosphate donor substrate as opposed to more sensitivity with the acceptor and/or divalent cation is an important possibility to explore partial reactions of the enzyme.

Acknowledgements

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