Regulation of *Escherichia coli* polynucleotide phosphorylase by ATP

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Polynucleotide phosphorylase (PNPase), an enzyme conserved in *Bacteria* and eukaryotic organelles, processively catalyzes the phosphorolysis of RNA releasing nucleotide diphosphates and the reverse polymerization reaction. In *E. coli* both reactions are implicated in RNA decay, as addition of either poly(A) or heteropolymeric tails targets RNA to degradation. PNPase may also be associated with the RNA degradosome, a heteromultimeric protein machine that can degrade highly structured RNA. Here we report that ATP binds to PNPase and allosterically inhibits both its phosphorolytic and polymerization activities. Our data suggest that PNPase-dependent RNA tailing and degradation occur mainly at low ATP concentrations, whereas other enzymes may play a more significant role at high energy charge. These findings connect RNA turnover with the energy charge of the cell and highlight unforeseen metabolic roles of PNPase.

Polynucleotide phosphorylase (PNPase), a polynucleotide nucleotidyl transferase (E.C. 2.7.7.8), is a homotrimeric enzyme involved in RNA turnover in *Bacteria* and eukaryotic organelles (1). PNPase processively catalyzes the phosphorolysis of RNA in 3' to 5' direction, thus releasing nucleoside diphosphates, and the reverse 5' to 3' template independent polymerization of nucleoside diphosphates (2-7). PNPase also binds RNA via its KH and S1 RNA binding domains located at the C-terminus of the polypeptide (6,8-11).

The monomeric subunit exhibits a five-domain structure that is widely conserved from bacteria to plants and mammals (12,13). The structural core of the subunit appears to be a duplication of an RNase PH (RPH) domain, with the two RPH-like domains connected by a poorly conserved linker domain. In the doughnut-shaped homotrimeric protein the subunits form a central channel where catalysis is thought to occur; the KH and S1 RNA binding domains are located on top of the enzyme, with the former immediately above the central channel and the latter facing outward away from the channel (14).

PNPase was originally implicated in the synthesis of cellular RNA before the template-dependent RNA polymerase was discovered (2,15,16); later on, due to its phosphorolytic activity, it was implicated in RNA degradation (6). It has long been assumed that, because of the high inorganic phosphate (Pi) intracellular concentration, PNPase would act *in vivo* mainly phosphorolytically (17). More recently, however, it was shown that PNPase can add heteropolymeric tails to RNA 3'-ends (18,19). As in *Escherichia coli* heteropolymeric tailing as well as polyadenylation performed by polyadenylpolymerase (PAP) target bacterial RNAs to degradation (20), both phosphorolysis and polymerization reactions participate in PNPase-dependent RNA decay. Finally, it was suggested that PNPase plays a central role in the biosynthetic pathway of dCTP by providing the CDP precursor (21), thus linking RNA turnover to DNA replication.
Although widely conserved in *Bacteria* and *Eukarya*, the *pnp* gene does not seem to be essential for survival. In some bacteria such as *E. coli* and *Yersinia enterocolitica*, however, it is essential for bacterial growth in the cold (22-24). PNPase seems also to be directly or indirectly involved in the control of several processes such as cold shock response (25) in *E. coli* and virulence in *Salmonella enterica* and *Yersinia* spp. (26,27). In plant chloroplast and mitochondria PNPase directly controls RNA stability via both degradation and tail addition (19,28). In the last few years human PNPase, which localizes in the mitochondrial inner membrane space and possibly in the cytoplasm, has been implicated in maintaining mitochondrial homeostasis, cell differentiation and senescence (29).

In *E. coli* PNPase has been localized both in the cytoplasm and associated to the cell membrane and ribosomes (30,31). In addition PNPase may exist as a single homotrimeric enzyme, associated with the RNA helicase RhlB (32), and in a multiprotein machine, the RNA degradosome, together with the endoribonuclease RNase E, which also serves as a scaffold for the assembly of the complex, RhlB, and enolase. In the RNA degradosome PNPase can degrade otherwise refractory double-stranded RNA regions in an ATP-dependent manner (33-35,36). In such heteromultimeric associations, PNPase can degrade otherwise refractory double-stranded RNA regions in an ATP-dependent manner (33-35,36). In such heteromultimeric associations, PNPase can degrade otherwise refractory double-stranded RNA regions in an ATP-dependent manner, a property that has been used as a functional degradosome assay (37).

In experiments aimed at assessing the functionality of RNA degradosomes from *pnp* mutants isolated in our laboratory (38), we realized that ATP concentrations higher than 5 mM retarded the appearance of the cleavage products and, in control reactions with purified PNPase, inhibited RNA degradation. Here we report that ATP binds to PNPase and inhibits both its phosphorolytic and polymerization activities. Such a direct control by ATP highlights an unforeseen metabolic role of PNPase that connects RNA turnover and the energy charge of the cell.

**EXPERIMENTAL PROCEDURES**

PNPase kinetic assays. PNPase was purified as described (39). Phosphorolytic activity of purified PNPase using poly(A) as a substrate was measured in a coupled pyruvate kinase/lactate dehydrogenase assay as described (40) at the indicated MgCl₂ concentrations. The assay, which measures the ADP produced as NADH oxidation, was performed in a 1-ml reaction mixture at 28°C and absorbance was continuously recorded at 340 nm. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol ADP/min under the working conditions.

PNPase polymerization activity was detected by measuring the Pᵢ released during the polymerization reaction as described (41) using the Enzchek Phosphate assay Kit (Invitrogen). Assays were performed at 25 °C in 1 ml volume containing 50 mM Tris-HCl, pH 7.5, 0.2 mM ADP, 20 µg/ml poly(A) and MgCl₂ at the indicated concentrations.

Degradation and 3’-tailing of PNPL1 RNA. For degradation and polymerization assays with a specific RNA substrate, ³²P-labelled PNPL1 RNA was synthesized by in vitro transcription with T7 RNA polymerase and [α-³²P]CTP using a DNA template obtained by PCR amplification of plasmid pAZ101 (11) with oligonucleotides FG676(5’-ctaatagctactatagggATGAATGATCT TCCGGTG; lower case letters, T7 promoter; uppercase letters, *E. coli*) and FG1387(5’-AATGTAATATCCTTTCTTTCTT TCCGTTGC; lower case letters, T7 promoter; uppercase letters, PNPL1 RNA encompasses the first 158 nt of the non-processed *pnp* transcript from the *pnp-p* promoter.

Phosphorolysis was performed at 26 °C in 30 µl reaction buffer (10 mM Tris HCl, pH 7.4; 0.75 mM DTT; 4.5 mM Mg acetate; 10 mM KCl) containing 0.2 µM PNPL1 (35000 cpm), 8.6 nM PNPase, and 10 mM Pᵢ. Polymerization was performed in the same conditions with 1 mM ADP and omitting Pᵢ. Reactions were stopped by adding 3 µl samples to 5 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.02% xylene cyanol) and heating for 5 min at 95 °C. The samples were analysed by denaturing 6% acrylamide gel electrophoresis and the autoradiographic image was acquired by phosphorimaging the excised gel.

**Photo-affinity labelling.** Photo-cross linking assay was performed by UV-irradiating (2 Jcm⁻²; 254 nm) 2 pmol of purified proteins pre-incubated 10 min at 21 °C with 3.3 pmol of either [α-³²P]ATP or [γ-³²P]ATP in 10 µl of 50 mM Tris-HCl pH 7.5, 0.4 mM DTT, in a UV-Stratalinker 2400 apparatus (Stratagene). 10 mM MgCl₂ and 33 µM ADP were also present where indicated. Before UV-irradiation, the samples were transferred in a 96-wells microtiter plate on ice. The UV-irradiated samples were then run on 10% SDS-PAGE, blotted onto a nitrocellulose filter and visualized by phosphorimaging.
RESULTS

ATP inhibits both PNPase enzymatic activities. While performing functional assays with RNA degradosomes assembled with mutant PNPases isolated in our laboratory (38), we observed that ATP concentrations higher than 5 mM retarded the appearance of degradation products (data not shown). To discriminate whether ATP directly inhibited the catalytic activity of PNPase rather than other properties of the entire degradosome, we tested the effect of ATP on purified PNPase by monitoring the shortening and the elongation of a specific RNA (PNPL1) substrate in the presence of Pi and ADP, respectively. PNPL1 is predicted to form a long stable stem-loop at the 5'-end followed by a less structured 3' tail (Fig 1A). As shown in Fig 1B, incubation of [32P]PNPL1 with PNPase in the presence of Pi led to the accumulation of a ca. 100 nt long degradation product, whereas the same substrate in the presence of ADP was elongated up to ca. 1 kb after 30 min incubation. Both phosphorolysis and polymerization, however, were completely inhibited by 10 mM ATP.

To rule out that Pi and ADP, which are spontaneous ATP hydrolysis products, could be responsible for these inhibitory effects by driving in the opposite direction the reversible reactions, we performed the PNPase assays in 1 mM ADP and varying Pi concentrations. As shown in Fig 1C, Pi up to 0.3 mM did not reverse the polymerization reaction with 1 mM ADP, while 1 mM ADP did not reverse degradation with 10 mM Pi. Thus contamination of Pi and ADP up to 3% and 10%, respectively, of the ATP concentration used in the previous experiment would be uninfluential on the PNPase reactions under our assay conditions.

By varying ATP concentration, we observed that under our assay conditions 4 mM ATP substantially inhibited both phosphorolytic and polymerizing activities (Fig 1D).

PNPase binds ATP. To test whether PNPase could directly bind ATP we performed affinity labelling experiments by UV-cross linking radiolabeled ATP to PNPase. As shown in Fig 2A, both [α-32P]ATP and [γ-32P]ATP (0.33 μM) were cross linked to PNPase. This indicates that ATP, rather than its spontaneous hydrolysis products ADP, AMP, or Pi, was cross linked to PNPase. No cross linking could be detected with carboxypeptidase used as a control, whereas a weak signal (barely visible in the original scan) in the absence of MgCl2 was detectable with ribosomal protein S1. 10 mM MgCl2 reduced but did not abolish cross linking. Affinity labelling was not competed by 33 μM ADP. Interestingly, however, cross linking was essentially not competed by cold ATP up to a ca. 100 μM concentration (Fig 2B and C), thus suggesting that 0.33 μM ATP was far from saturation, in keeping with the I0.5 values determined for the nucleotide (see below).

ATP allosterically inhibits PNPase. To perform kinetic analysis of PNPase phosphorolytic activity at different ATP concentrations we used poly(A) as a substrate and measured the appearance of ADP in a coupled pyruvate kinase/lactate dehydrogenase assay at constant, saturating (30 μg/ml) poly(A) and at varying Pi concentrations. The results (Fig 3A) were consistent with a mixed-type inhibition (42), with a scanty increase in Km and a substantial decrease in Vmax on increasing ATP concentrations. Thus the pattern observed is close to pure non-competitive inhibition. In particular, Vmax dropped from about 0.7 to 0.2 U/mg when ATP was raised from zero to 5 mM. Similar results were obtained at fixed Pi and varying poly(A) concentrations. However, at poly(A) concentrations close to or lower than the Km (about 2 μg/ml), measurements were subject to large dispersions, which prevented us from accurately determining the kinetic parameters (data not shown). In any case, the activity was completely abolished even at 30 μg/ml poly(A) (data not shown), thus ruling out competitive inhibition. We therefore conclude that the ATP-binding site is distinct from that of both substrates.

According to the kinetic model, replots of slopes and 1/v-axis intercepts as a function of inhibitor concentration should yield straight lines whose intercepts on the abscissa equal the inhibition constant (42). However, following this procedure we observed, surprisingly, large and systematic deviations from linearity of the experimental points. We thus conclude that PNPase does not conform to a classical mixed-type or non-competitive inhibition model. Moreover, when plotting the percentage of PNPase inhibition versus ATP concentration at saturating substrates, a sigmoidal curve was obtained (Fig 3B), which clearly points to cooperative, allosteric binding of the nucleotide. Based on the best-fitting curve, a Hill coefficient of 2.3 and a I0.5 (i.e., a concentration that gives 50% inhibition) of 3.3 mM were estimated. The
experiments were performed at 5 and 13 mM MgCl₂ and the results obtained in either condition were superimposable (Fig 3B). This rules out the hypothesis that the inhibition observed simply depends on depletion of Mg²⁺ (an ion required for PNAse activity) complexed by ATP. A similar profile was obtained with purified FLAG-Rne degradosome (43) rather than trimeric PNPase (data not shown).

We also checked the effect of other nucleoside triphosphates on PNPase activity. Our results (Table 1) show that the purine nucleotides GTP, dGTP, dATP and non-hydrolyzable ATP were also inhibitory, whereas pyrimidine nucleotides CTP and UTP did not exert any appreciable effect.

To detect the effect of ATP in the synthetic direction, we measured the released Pi using a commercially available enzymatic assay for phosphate detection that allows continuous measurements of PNPase activity. The data shown in Fig. 3C indicate that ATP also inhibits polymerization at both 5 and 13 mM with I₀.₅ values of about 5 mM.

**DISCUSSION**

Here we present clear-cut evidence, using both a model RNA and poly(A) as substrates, that *E. coli* PNPase binds to and is inhibited by ATP. Although this effect is likely to play a major regulatory role, no such observation was reported to date despite over five decades of investigations on this enzyme.

Our results show that the ATP-binding site is distinct from that of the substrates, as none of them could abolish or reduce the inhibitory effect even at saturating concentrations. In particular, our kinetic analysis demonstrates a mixed-type inhibition of ATP towards Pᵢ. Furthermore, replots of slopes and 1/v-axis intercepts as a function of inhibitor concentration did not yield straight lines as predicted by the classical mixed-type inhibition model (42). In contrast, the sigmoidal profile of PNPase inhibition as a function of ATP concentration indicates a cooperative, allosteric binding of the nucleotide. In particular, we assessed a Hill coefficient of 2.3, well in agreement with the trimeric structure of the enzyme, and a I₀.₅ of 3.3 mM. Similar results were obtained at a poly(A) concentration close to the Kᵢ, i.e. 2 µg/ml, and also using the whole degradosome instead of PNPase alone (data not shown). This latter observation implies that ATP may also modulate PNPase function at the level of the multi-protein complex. The nucleotide also exerted a comparable inhibition on the synthetic activity, with a Hill coefficient of 2.3 as in the phosphorolytic direction, and a I₀.₅ of 5.0 mM. Our kinetic assays may not fully reproduce the physiological conditions, as ADP and Pᵢ are promptly removed by the auxiliary reactions in the phosphorolytic and synthetic tests, respectively. This, however, should not affect the mode of ATP inhibition, since the ATP binding site is distinct from that of the substrates.

Taken together, these results strongly support the idea that the inhibition exerted by ATP on PNPase is a phenomenon of physiological relevance. In particular, enzymes displaying a sigmoidal dependence of their activity on metabolite concentrations are believed to exert major roles in metabolic regulation. However, the interpretation of our observations is not straightforward. Classical studies conducted by Atkinson several decades ago, established that high values of energy charge, and thus high ATP and low ADP and AMP, inhibit the catabolic pathways in both eukaryotes and prokaryotes (44,45). Unfortunately accurate assessment of the enzyme behavior under physiological conditions is not feasible as the local, intracellular concentration of Pᵢ and nucleotides can be hardly predicted. Nevertheless our finding may help to shed light on the physiological significance of different RNA degradation pathways operating in *E. coli*.

It has been pointed out that phosphorolytic degradation of mRNA by PNPase, which operates close to equilibrium, is more energy-saving than the hydrolysis performed by RNase II, which releases nucleoside monophosphates and is far from equilibrium (46). Moreover, 3’-end tailing of RNA may be performed both by PAP at the expenses of ATP and by PNPase, which uses NDPs. Thus PNPase-dependent degradation of RNA would be energetically more favourable than RNase II/PAP-dependent degradation and would be activated at a low energy charge. Additional regulatory levels might occur in the RNA degradosome and in the PNPase-RhlB complex, where the RNA helicase unwinds RNA secondary structures and hydrolyzes ATP (35). This could lower the local ATP concentration with concurrent PNPase activation. Conversely, a low helicase activity may allow a local increase of ATP concentration thus inhibiting RNA degradation. Predictions of the above speculations are that RNase II, the PNPase-RhlB complex and the RNA degradosome may perform RNA degradation at high energy charge, whereas free

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PNPase activity would be more relevant at low ATP concentrations.

Although information on RNA decay mechanisms at different energy charge is scanty, an interesting observation that supports our model has been done by Andrade et al. (47). These authors found that PNPase strongly contributed to the decay rate of ompA transcript in stationary phase, whereas RNase II seemed to minimally affect the half life of this mRNA. A systematic study of the role of different phosphorolytic and hydrolytic exoribonucleases on the mRNA decay at different energy charge will help understanding the physiological role of PNPase inhibition by ATP.

The above interpretation does not rule out other possible scenarios that implicate PNPase in metabolic processes other than the mere RNA decay. For instance, the release of ADP and other NDPs at low energy charge with concomitant formation of high energy, phosphoanhydride bonds, might be a mechanism that exploits RNA degradation to reconstitute ATP via adenylate kinase. Finally, as PNPase may have a role in providing the dCTP precursor CDP (21), inhibition by ATP might play a role in coordinating DNA replication, RNA degradation, and cell energy charge.

Whatever may be their interpretation, our findings imply that a reconsideration of the precise physiological role(s) of this enzyme is required. The accurate determination of the intracellular concentrations of the chemical species affecting PNPase activity (NTPs, NDPs and P) and the prevalence of alternative RNA degradation pathways under different physiological conditions such as metabolic stress and cold shock, might provide clues to the understanding the role of the ATP inhibitory effect here reported. Moreover, it will be interesting to assess whether this property is shared by other PNPases and whether it controls the pleiotropic effects associated with this enzyme in bacteria, plants and humans.

**BIBLIOGRAPHY**


**FOOTNOTES**

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† These authors contributed equally to this work

The abbreviations used are: DTT, dithiothreitol; EDTA, ethylene diamino tetraacetic acid; PAP, polyadenylpolymerase; Pi, inorganic phosphate; pnp, gene encoding PNPase; PNPase, polynucleotide phosphorylase; PNPL1, pnp leader RNA; RPH, RNase PH
FIGURE LEGENDS

Fig 1. PNPase inhibition by ATP. The assays were performed with a uniformly radiolabelled PNPL1 RNA as described in Methods. A) MFOLD (48) computed secondary structure of PNPL1 showing a 99 nt long hairpin structure at the 5’ end and a looser conformation of the 3’ region. The two bases preceding the 5’-end of pnp-p RNA are lower case. B) Degradation (10 mM Pi) and polymerization (1 mM ADP) reactions without or with 10 mM ATP. Lower case letters from a to e indicate the reaction times 0, 2.5, 5, 10, 30 min, respectively. The arrowheads point to PNPL1 (P), the ca. 100 bases long stem-loop degradation (SL) and the ca. 1 kb long elongation (E) products. C) Polymerization reaction was performed in the presence of 1 mM ADP and increasing Pi concentrations, as indicated on top of the lanes, and quenched after 30 min. -: no PNPase added. D) Degradation (10 mM Pi) and polymerization (1 mM ADP) reactions at increasing ATP concentrations.

Fig 2. Affinity labeling of PNPase by UV-crosslinking with 32P-ATP. The analysis was performed as described in Methods. A) 2 pmol of each protein indicated over the lanes were incubated in 10 μl reactions with 0 or 10 mM MgCl2 and 3.3 pmol of either [α-P32]ATP or [γ-P32]ATP as indicated. 33 μM ADP was also added in the first lane reaction. P, PNPase, C, bovine pancreas carboxypeptidase; S, E. coli His-tagged ribosomal protein S1. B) 2 pmol PNPase were incubated in 10 μl reactions with 3 pmol of [α-P32]ATP and unlabelled ATP at the concentration indicated over the lanes. C) The relative intensity (RI) of the signals in Fig 2B was plotted against the cold ATP concentration.

Fig 3. Kinetic analysis of PNPase inhibition by ATP. A) Double-reciprocal plots of PNPase phosphorolytic activity at varying Pi concentrations. The activity was assayed at 28 °C as described in Methods. ATP concentrations were 0 mM (▲), 2 mM (●), and 5 mM (■). Each point is the mean of at least four independent determinations. Vertical bars represent standard deviations. B) Inhibitory effect of ATP towards PNPase phosphorolytic activity. The activity was assayed at 28 °C in the presence of 30 μg/ml poly(A), 10 mM Pi, and either 5 (open symbols) or 13 mM MgCl2 (closed symbols) and otherwise as described in Methods. Inhibition is expressed as percentage of the samples without ATP. Each point is the mean of at least four independent determinations. Vertical bars represent standard deviations. Inset: Hill plot of the ATP inhibitory effect. Abscissa: log[ATP]; ordinate: log(f/1-f), where f represents the fraction inhibited. C) Inhibitory effect of ATP towards PNPase synthetic activity. The activity was assayed at 25°C in the presence of 0.2 mM ADP, 20 μg/ml poly(A) and either 5 (open symbols) or 13 mM MgCl2 (closed symbols) and otherwise as described in Methods. Each point is the mean of at least four independent determinations. Vertical bars represent standard deviations.
Table 1. Inhibitory effect of different nucleoside triphosphates towards PNPase phosphorolytic activity.

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<th>ATP</th>
<th>dATP</th>
<th>β, γ-imido-ATP</th>
<th>GTP</th>
<th>dGTP</th>
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<td>Residual activity (%)</td>
<td>30</td>
<td>39</td>
<td>50</td>
<td>65</td>
<td>40</td>
<td>102</td>
<td>98</td>
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a Assays were performed at 28°C as described in Methods in the presence of 5 mM nucleotide to be tested and 13 mM MgCl₂.

b The activity (average of at least three independent measurements) is expressed as percentage of the control assays without nucleotide added.
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