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Short communication

The mechanism of the polynucleotide phosphorylase-catalyzed arsenolysis of ADP

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ABSTRACT

Using ADP and arsenate (AsV), polynucleotide phosphorylase (PNPase) catalyzes the apparent arsenolysis of ADP to AMP-arsenate and inorganic phosphate, with the former hydrolyzing rapidly into AMP and AsV. However, in the presence of glutathione, AMP-arsenate may also undergo reductive decomposition, yielding AMP and arsenite (AsIII). In order to clarify the mechanism of ADP arsenolysis mediated by *Escherichia coli* PNPase, we analyzed the time course of the reaction in the presence of increasing concentrations of ADP, with or without polyadenylate (poly-A) supplementation. These studies revealed that increasing supply of ADP enhanced the consumption of ADP but inhibited the production of both AMP and AsIII. Formation of these products was amplified by adding trace amount of poly-A. Furthermore, AMP and AsIII production accelerated with time, whereas ADP consumption slowed down. These observations collectively suggest that PNPase does not catalyze the arsenolysis of ADP directly (in a single step), but in two separate consecutive steps: the enzyme first converts ADP into poly-A, then it cleaves the newly synthesized poly-A by arsenolysis. It is inferred that one active site of PNPase can catalyze only one of these reactions at a time and that high ADP concentrations favor poly-A synthesis, thereby inhibiting the arsenolysis.

1. Introduction

Polynucleotide phosphorylase (PNPase; polyribonucleotide: orthophosphate nucleotidyltransferase; EC 2.7.7.8) is a ubiquitous enzyme long known for its RNA-processing activity [1,2]. However, the multifunctionality of this enzyme is highlighted by recent findings which suggest that PNPase is also involved in RNA import into mammalian mitochondria [3], and it may contribute to DNA repair in *Bacillus subtilis* by degrading single stranded DNA from the 3′-end [4]. As an RNA-processing enzyme, PNPase catalyzes the polymerization of nucleoside diphosphates into polyribonucleotides with concomitant formation of inorganic orthophosphate (P_i), whereas in the reverse mode, it cleaves the 3′ terminal nucleotides from polyribonucleotide chains (RNA) using P_i (termed phosphorolysis). In addition, PNPase can also catalyze the exchange of the terminal phosphate group of ADP and P_i.

Owing to its high structural similarity to P_i, inorganic arsenate (AsV) can readily replace P_i in these reactions. Therefore, PNPase can catalyze the arsenolysis of RNA, including the homopolymeric RNA variant polyadenylate (poly-A), and can exchange the β phosphate of ADP for AsV, which is called arsenolysis of ADP [5]. Both of these reactions yield AMP-arsenate, a mixed anhydride [6]. Ester and especially anhydride derivatives of AsV are labile and undergo rapid hydrolysis. However, if a thiol compound, such as glutathione (GSH), is present, a fraction of the arsenate ester or anhydride escapes hydrolysis and decomposes reductively, yielding the trivalent arsenical arsenite (AsIII) [7]. Thus, AMP-arsenate formed by the PNPase-catalyzed arsenolytic reactions can decompose into AMP and AsV or, in presence of GSH, also into AMP and AsIII. Arsenolysis of ADP in the presence of a thiol should produce AMP and AsIII in proportionate quantities; however, formation of AMP should exceed that of AsIII because AMP would originate from both the hydrolytic and the reductive decomposition of AMP-arsenate.

The mechanism whereby PNPase catalyzes the replacement of the β phosphate of ADP by AsV is still unclear. Theoretically, it can proceed (1) directly, by a true exchange, or (2) indirectly, by two sequential reactions, in which polymerization of ADP into poly-A precedes the arsenolysis of the *de novo* synthesized poly-A [2,5,8,9]. Serendipitously, when studying the role of PNPase in thiol-mediated AsV reduction [6], we made observations arguing for one of the mechanisms. These findings, reported below, originate from

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studies on the arsenolysis of ADP by PNPase in the presence of GSH. In these experiments, we determined the time courses of ADP consumption as well as AMP and AsIII production when *Escherichia coli* PNPase was supplied with increasing concentrations of ADP in the presence or the absence of trace amounts of poly-A.

2. Materials and methods

His_6-tagged, wild-type polynucleotide phosphorylase (PNPase) was purified from *E. coli* GF5322 strain according to Matus-Ortega et al. [10] and was assayed by the spectrophotometric method of Ghetta et al. [11], as recently described in more detail [6].

The arsenolysis of ADP by PNPase was followed by measuring the formation of AMP and AsIII. For comparison, the disappearance of ADP was also assessed. For this purpose, *E. coli* PNPase (50 mU/ml) was pre-incubated for 5 min at 37 °C with GSH (10 mM) in 100 mM Tris buffer (pH 7.7) containing 5 mM $MgCl_2$. Then, ADP and AsV (200 μM) were added to start the incubation. Immediately before terminating the incubations, the reaction mixtures were divided into two samples. In the sample to be used for AsIII and AsV analysis, the reaction was stopped by addition of 1/3 volumes of 50 mM $CdSO_4$ followed by 1/3 volumes of 1.5 M perchloric acid containing 50 mM $HgCl_2$. The rationale for this procedure is given elsewhere [12]. In the sample destined for AMP and ADP analysis, the reaction was terminated by adding 1/6 volume of 2.1 M perchloric acid. After these treatments, the samples were stored at −70 °C until analysis.

The analyses were performed on the 10,000 g supernatants of the perchloric acid-treated samples. AsIII and AsV were separated and quantified by HPLC-HG-AFS, as detailed elsewhere [13,14]. ADP and AMP were quantified by reverse phase HPLC analysis with spectrophotometric detection at 254 nm, as described [6]. When calculating the PNPase-mediated AsIII formation, the small amount of AsIII originating from spontaneous reduction of AsV by GSH was routinely determined and subtracted from that measured in the presence of both PNPase and GSH.

3. Results and discussion

Theoretically, PNPase can catalyze the exchange between the terminal phosphate group of ADP and AsV by two ways. The first is the direct replacement of the phosphate in ADP by AsV:

$$\text{ADP} + \text{AsV} \leftrightarrow \text{AMP-arsenate} + P_i \quad (1)$$

The following observations do not support this mechanism. When the initial 0.25 mM ADP concentration was increased two- and four-fold, the rates of both AsIII and AMP formation decreased roughly in inverse proportion to the ADP concentration (Fig. 1, A and B, without poly-A). Conversely, the rate of ADP consumption increased with elevation of the ADP level (Fig. 1, C). Addition of 10 μg/ml poly-A alleviated the ADP-induced declines in AMP and AsIII formation, but accentuated the simultaneous ADP consumption (Fig. 1). It should be stressed that such poly-A concentration is far from saturating in the arsenolytic reaction [6], although it is largely saturating in the conventional phosphorolytic reaction [10]. If PNPase catalyzed the arsenolysis of ADP directly, one would have expected either an increase in the rates of AMP and AsIII formation upon an increase in ADP concentration, or no change if the enzyme is saturated.

The time courses of AMP and AsIII production as well as of ADP consumption also fail to support reaction (1) as the mechanism for arsenolysis of ADP. As demonstrated in Fig. 2 (A and B), the amount of AMP and AsIII formed increased more than proportionately to the incubation time, while the amount of ADP consumed increased much less than proportionately to the incubation time (Fig. 2, C), indicating that production of AMP-arsenate, and so of AMP and

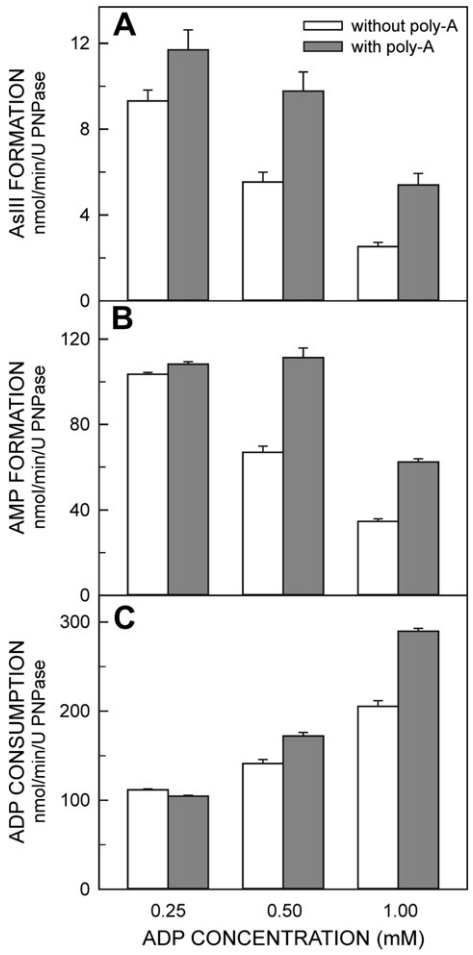

Fig. 1. PNPase-mediated arsenolysis of ADP as a function of ADP concentration. *E. coli* PNPase (50 mU/ml) was pre-incubated at 37 °C for 5 min with GSH (10 mM) and poly-A (0 or 10 μg/ml) in 100 mM Tris buffer (pH 7.7) containing 5 mM $MgCl_2$. Then ADP (at concentrations indicated) and AsV (200 μM) were added in rapid succession to start the 30-minute incubation. Bars represent the rates of AsIII (panel A) or AMP (panel B) formation, or ADP consumption (panel C) in three incubations (mean ± SEM).

AsIII, accelerates with time, while consumption of ADP decelerates. Data derived from Fig. 2 support this conclusion: during the first, second, and third 15-min period of the incubation with ADP plus poly-A approximately 98, 195, and 298 nmol/U AsIII was formed, 1450, 1890, and 1760 nmol/U AMP was produced, while 3700, 1460, and 535 nmol/U ADP was consumed, respectively. It is important to note that the concentration of ADP during the incubations (0.32, 0.24, and 0.21 mM at 15, 30, and 45 min, respectively) well exceeded its K_M under these conditions (approximately 0.1 mM; our unpublished observation). Thus, the precipitous decline of ADP consumption can only be marginally ascribed to ADP shortage. Furthermore, if ADP arsenolysis occurred directly, it would be expected that AsIII and especially the AMP formation is proportional to the ADP consumption.

Alternatively, PNPase may catalyze the arsenolysis of ADP in two consecutive reactions:

$$\text{ADP} \leftrightarrow \text{Poly-A} + P_i \quad (2)$$

$$\text{Poly-A} + \text{AsV} \leftrightarrow \text{AMP-arsenate} \quad (3)$$

The observation that the increased ADP supply resulted in increased ADP consumption (Fig. 1, C) but decreased formation of AMP and AsIII (i.e., AMP-arsenate; Fig. 1, A and B) is not compatible