Phosphorylation of *Escherichia coli* poly(A) polymerase I and effects of this modification on the enzyme activity

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Abstract
In *Escherichia coli*, RNA polyadenylation is catalyzed mainly by poly(A) polymerase I (PAP I). Here we demonstrate that a PAP I variant with a C-terminal His tag (PAP I-His) can be phosphorylated both *in vivo* and in an artificial *in vitro* system. The *in vivo* phosphorylation of PAP I-His impairs activity of this enzyme. Previous studies, performed by others, indicated that phosphorylation of His-tagged proteins usually reflects such a modification of their native counterparts in bacterial cells. Therefore, our results suggest that phosphorylation and dephosphorylation of PAP I may be important regulatory processes in the control of activity of this enzyme.

Introduction
The process of RNA polyadenylation at its 3′ end occurs in both eukaryotic and prokaryotic cells (Sarkar, 1996; Carpousis *et al*., 1999; Proudfoot & O’Sullivan, 2002; Kushner, 2004; Gilmartin, 2005). However, in contrast to eukaryotes, polyadenylation of bacterial RNA causes a decrease in stability of transcripts (Kushner, 2004).

Poly(A) polymerase I (PAP I), encoded by the *pcnB* gene (Cao & Sarkar, 1992), is the main enzyme responsible for RNA polyadenylation in *Escherichia coli* (O’Hara *et al*., 1995; Mohanty & Kushner, 1999). As degradation of transcripts plays a major role in the control of bacterial gene expression, and addition of A residues at the 3′ end of prokaryotic RNA results in rapid degradation of these molecules, regulation of activity of PAP I would be expected. However, until recently, mechanisms controlling *pcnB* expression remained obscure. Recent studies revealed that production of PAP I is regulated at the levels of both transcription (Jasiecki & Węgrzyn, 2006) and translation (Binns & Masters, 2002). Moreover, efficiencies of RNA polyadenylation, *pcnB* gene transcription and PAP I synthesis were found to be in reverse correlation to *E. coli* growth rate, indicating a polyadenylation-mediated influence of physiological conditions on the regulation of gene expression in bacteria (Jasiecki & Węgrzyn, 2003). PAP I was found to be localized in both cytoplasm and cellular membranes, suggesting that specific localization of this protein may also be important in the control of its activity (Jasiecki & Węgrzyn, 2005).

Overexpression of PAP I is toxic to *E. coli* (Binns & Masters, 2002), strongly suggesting that production and/or activity of this protein must be precisely regulated. Although specific mechanisms of regulation of the *pcnB* gene expression were reported, as mentioned above (Binns & Masters, 2002; Jasiecki & Węgrzyn, 2003, 2006), little is known about the control of PAP I enzyme activity. One of the most common mechanisms of regulation of protein activity is specific phosphorylation. In fact, human PAP has been shown to be phosphorylated (Thuresson *et al*., 1994; Colgan *et al*., 1996). Therefore, here we asked whether *E. coli* PAP I can be phosphorylated, and if so, how this modification affects the enzyme.

Materials and methods

**Bacteria and plasmids**

*Escherichia coli* strain JM109 was used. Plasmid pBR322 was used for PCR- and *in vitro* transcription-mediated production of RNA I. These commonly used prokaryotic tools are described by Sambrook *et al*. (1989). For construction of a plasmid overexpressing the *pcnB* gene, a fragment of *E. coli*...
chromosome bearing this gene was PCR amplified using following primers: pcnEco, 5' TAG AAT TCA TGC TGA GCT ATG ATT AGC CGC, and pcnkon, 5' CAT AGA TCT TGC GGT ACC CTC ACG ACG TGG. The amplified fragment (1478 bp), containing EcoRI and BglII sites (introduced during PCR amplification), was digested with these enzymes and cloned into corresponding sites of the pQE-60 vector (Qiagen). The constructed plasmid, called pQEPAK, contains the pcnB gene fused to a sequence coding for His6 (this fusion encodes the PAP I-His protein), located downstream of the T5 promoter/lac operator element.

**Proteins**

The PAP I-His protein was purified from the *E. coli* JM109/pQEPAK strain induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) [bacterial cells were collected at the late exponential phase of growth in LB medium (Sambrook *et al.*, 1989) and used for protein isolation] using Ni-NTA Spin Columns (Qiagen), according to the manufacturer’s instruction. The catalytic domain of *Bacillus subtilis* PrkC kinase, called PrkCc, has been described previously (Madec *et al.*, 2002) and was kindly provided by Dr M. Obuchowski (Medical University of Gdańsk, Poland). NtrC protein and σ24 subunit of *E. coli* RNA polymerase were obtained from A. Janaszak (University of Gdańsk, Poland), and σ70-containing holoenzyme of *E. coli* RNA polymerase was obtained from Dr A. Szalewska-Palasz (University of Gdańsk, Poland). Recombinant human α-L-iduronidase was from Genzyme. Shrimp Alkaline Phosphatase was purchased from Promega.

**In vitro protein phosphorylation**

Indicated amounts of various purified proteins were mixed with 1 μg of PrkCc in buffer K (50 mM HEPES pH 8.0, 5 mM MgCl2). One micro-Curie of [γ-32P]ATP (3000 Ci mmol−1) was added and the mixture was incubated at 30 °C for 20 min. The reaction was stopped by addition of the lysis buffer [125 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% sucrose, 1% β-mercaptoethanol, 0.2% bromophenol blue], the samples were boiled and proteins were separated electrophoretically [(sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)]. Following electrophoresis, gels were washed in 10% trichloroacetic acid at 80 °C for 30 min and analyzed using a phosphorimager (Molecular Imager FX, BioRad).

**In vivo protein phosphorylation**

Phosphorylation of proteins in bacterial cells was performed according to a previously described procedure (Klein *et al.*, 2003). Briefly, *E. coli* cultures were grown in LB medium (Sambrook *et al.*, 1989) at 37 °C to an OD600 of 0.2. Cultures were harvested by centrifugation (2000 g, 5 min), washed twice with a minimal medium lacking phosphates, diluted in this medium 10 times and grown to OD750 of 0.2. Bacteria were labeled after addition of [32P]orthophosphate to the medium (final concentration 30 μCi mL−1) for 30 min. Following lysis of bacterial cells in the lysis buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 20% sucrose, 1% β-mercaptoethanol, 0.2% bromophenol blue), proteins were separated electrophoretically (SDS-PAGE), stained with Coomassie Brilliant Blue and analyzed using a phosphorimager (Molecular Imager FX, BioRad).

**Results and discussion**

We asked whether *E. coli* PAP I can be phosphorylated. It was demonstrated previously that this enzyme is toxic to...
E. coli when overproduced even in moderate amounts, and in fact, under standard laboratory growth conditions, PAP I is produced at a low level due to control processes operating at the stages of both transcription and translation (Binns & Masters, 2002; Jasiecki & Węgrzyn, 2006). Hence, it is difficult to monitor the pcnB gene product in vitro and to purify native PAP I in amounts sufficient for in vivo analyses. Therefore, we decided to study a His-tagged variant of PAP I in both in vivo and in vitro experiments.

To determine whether PAP I is a potential substrate in a phosphorylation reaction, an artificial system was used, in which a highly active catalytic domain of the Bacillus subtilis PrkC kinase, called PrkC (Madec et al., 2002), was employed. PrkC is able to phosphorylate its own molecules as well as to phosphorylate other proteins which bear a phosphorylation signal. In control experiments, several phosphorylated proteins were tested in the in vitro PrkC-mediated phosphorylation assay, including those susceptible and resistant to this kind of modification. As expected, PrkC was able to phosphorylate its own molecules as well as molecules of E. coli NtrC protein and the σ54 subunit of RNA polymerase. β/β' subunit(s) of E. coli RNA polymerase was/were also phosphorylated in this reaction whereas no signal corresponding to such a modification could be detected in the α-subunit of RNA polymerase and in human α-i-iduronidase (Fig. 1). In this assay we observed phosphorylation of PAP I-His (Fig. 1), suggesting that this protein may also be modified in E. coli cells.

We next tested whether in vivo phosphorylated PAP I-His can be recovered from growing cells. Because PAP I is not produced at high levels from a single copy of pcnB, we have cultured bacteria bearing a plasmid overexpressing this gene in a minimal medium, and after supplementation with inorganic 32P the cultures were harvested, and protein extracts were separated using SDS-PAGE. Gels were stained with Coomasie Brilliant Blue and then subjected to autoradiography. As demonstrated in Fig. 2, radioactive bands were observed at positions corresponding to overproduced PAP I-His (two bands of this protein are visible, according to previously published reports: Jasiecki & Węgrzyn, 2003; Mohanty et al., 2004). In control experiments no such radioactive signals were detected in extracts of cells bearing only the vector used for pcnB cloning, strongly suggesting that the radioactivity observed in lane 1 of the right panel of Fig. 2 comes from the modified PAP I-His.

If phosphorylation of PAP I-His plays a role in the regulation of activity of this enzyme, one should be able to detect differences in the efficiency of RNA polyadenylation catalyzed by the modified and the unmodified enzyme. To test this, an in vitro RNA polyadenylation reaction was performed using either untreated PAP I-His or PAP I-His treated with a nonspecific phosphatase. In this assay we observed significantly more rapid and more efficient polyadenylation of RNA I (one of known substrates for PAP I) by the de-phosphorylated enzyme relative to an untreated His-tagged PAP I (Fig. 3). It appears that the lower efficiency of the reaction catalyzed by the untreated enzyme [observed as a low intensity of the RNA I (A)n signal and an almost complete disappearance of unmodified RNA I molecules] resulted from production of shorter poly(A) tails that formed a hardly detectable smear on the gel (seen after overexposure of the gel; data not shown). Prolonged incubation of the reaction mixture, without electrophoresis, did not result in a disappearance of RNA I in any samples

![Fig. 1.](https://academic.oup.com/femsle/article-abstract/261/1/118/537171)

![Fig. 2.](https://academic.oup.com/femsle/article-abstract/261/1/118/537171)
This exclude a possibility that a relatively weak signals at RNA I (A)n and RNA I positions in lanes 11–14 (Fig. 3) was caused by a loss of RNA due to putative impurities that might be present in PAP I-His samples obtained from cell lysates by affinity chromatography. Moreover, in this light, it appears unlikely that the samples might contain impurities limiting polyadenylation unless themselves dephosphorylated.

In conclusion, we demonstrate that *E. coli* PAP I-His can be phosphorylated and this modification influences activity of this enzyme significantly. A de-phosphorylated form of PAP I-His is considerably more active than a phosphorylated form of this protein, producing longer poly(A) tails more rapidly. Although we used a His-tagged derivative of PAP I, it is likely that our results reflect phosphorylation of the native enzyme as it was demonstrated previously that His-tagged derivatives can be used as adequate models in studies on protein phosphorylation (see, for example, Castelli et al., 2003; Kamei et al., 2003; Sanowar & Le Moual, 2005). Although some functional differences between phosphorylated native proteins and their His-tagged counterparts were reported in a few cases, it appears that the main features of this modification are unaffected by addition of six His residues at the C-terminus (Du et al., 2005; Perron-Savard et al., 2005). Thus, we suspect that phosphorylation may play an important role in the control of activity of PAP I in *E. coli* cells. This can be especially important as the level of RNA polyadenylation has been shown to be crucial in regulation of gene expression in response to bacterial growth rate (Jasiecki & Węgryzn, 2003), but on the other hand, PAP I is toxic to *E. coli* when overproduced. Therefore, it appears that phosphorylation of PAP I may be a specific mechanism for reducing activity of this enzyme, in combination with the control of expression of its gene, *pcnB*. Since overproduced His-tagged PAP I was used in our experiments, it is rather hard to estimate the level of phosphorylation of the wild-type enzyme in *E. coli* cells and the degree to which this modification influences PAP I activity. We suspect that phosphorylation is an important mechanism in the regulation of RNA polyadenylation efficiency, but apparently this protein modification does not abolish PAP I activity completely. Finally, specific kinase(s) and phosphatase(s) involved in PAP I phosphorylation and dephosphorylation in *E. coli* cells remain to be determined.

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**References**


