Oligoribonuclease is a common downstream target of lithium-induced pAp accumulation in *Escherichia coli* and human cells

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ABSTRACT

We identified Oligoribonuclease (Orn), an essential *Escherichia coli* protein and the only exonuclease degrading small ribonucleotides (5mer to 2mer) and its human homologue, small fragment nuclease (Sfn), in a screen for proteins that are potentially regulated by 3′-phosphoadenosine 5′-phosphate (pAp). We show that both enzymes are sensitive to micromolar amounts of pAp *in vitro*. We also demonstrate that Orn can degrade short DNA oligos in addition to its activity on RNA oligos, similar to what was documented for Sfn. pAp was shown to accumulate as a result of inhibition of the pAp-degrading enzyme by lithium, widely used to treat bipolar disorder, thus its regulatory targets are of significant medical interest. CysQ, the *E.coli* pAp-phosphatase is strongly inhibited by lithium and calcium *in vitro* and is a main target of lithium toxicity *in vivo*. Our findings point to remarkable conservation of the connection between sulfur- and RNA metabolism between *E.coli* and humans.

INTRODUCTION

While the basic building blocks of the cell are usually considered when studying intermediary metabolism, some are overlooked. 3′-phosphoadenosine 5′-phosphate (pAp) in the metabolism of sulfur is a case in point. It is a ubiquitous 3′-phosphorylated nucleotide derived from sulfur assimilation. pAp is generated from phosphoadenosine 5′-phosphosulfate (PAPS), in an intermediate step in sulfate assimilation, by two different mechanisms, as a by-product of either (i) sulfonation reactions, or (ii) reduction to sulfite. Sulfonation, catalyzed by numerous sulfotransferases is the transfer of sulfate to a large number of acceptor molecules that play important roles in mammalian cells; in addition to its effects on structure and function of macromolecules, sulfonation is also involved in deactivation and bioactivation of hormones, neurotransmitters, xenobiotics and elimination of end products of catabolism (1). Reduction of PAPS to sulfite is part of the pathway that leads to the biosynthesis of cysteine or methionine in microorganisms, a pathway that is absent in humans.

Furthermore, and independent from sulfur metabolism, pAp is also generated from coenzyme A during the transfer of the 4-phosphopantetheine group to acyl carrier protein (ACP) in fatty acid synthesis or to secondary metabolites such as peptide antibiotics, surfactin or polyketides (2).

Regardless of how pAp is made, it needs to be recycled to AMP, which is accomplished by a 3′-nucleotidase. In *Escherichia coli*, CysQ plays this role. Mutants in *cysQ* require cysteine or sulfite for aerobic growth (3) and were therefore originally suggested to help control the pool of PAPS or a toxic derivative of it. Later the protein was shown to hydrolyze pAp *in vitro* (4). The question arises whether this nucleotide is solely a metabolic intermediate or whether it has regulatory function as well. pAp is known to act as competitive inhibitor of enzymes that use PAPS, mainly PAPS reductase and sulfotransferases (1) and to inhibit Xrn1p in yeast (5); however, other targets could also exist. The importance of the search for pAp targets is attested by the fact that the enzymes degrading pAp belong to a structurally conserved protein family that is uniquely inhibited by lithium (6). Lithium has been used for five decades to treat bipolar disorder, a severe and often life-threatening disease. Lithium proved to be an effective drug for this disorder and shows prophylactic as well as therapeutic effects on both the manic and the depressive phases, yet the molecular basis of its action is still not understood.

The inhibition of pAp-phosphatase by lithium occurs via an uncompetitive mechanism and is characterized by a low K_i of 0.3 mM (7). This value lies below the therapeutic concentration range (0.6–1.2 mM) and defines pAp-phosphatase as the most sensitive lithium target described in the literature so far. Physiological significance of this inhibition is inferred from

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the demonstration of pAp accumulation to millimolar range in yeast treated with lithium (8). Moreover, recent data show direct evidence that it is this accumulation of pAp that mediates the toxic effect of lithium in yeast (9). It is not known if this correlation is evolutionary conserved and how pAp causes toxicity. pAp could therefore account for some of the toxic effects of lithium administration in humans treated for bipolar disorder. In addition it could also account for some of the therapeutic effects. For this reason, we were interested in investigating regulatory targets of pAp, starting from the model bacterium *E.coli*. Among the proteins identified are the oligoribonuclease from *E.coli* and its human homolog small fragment nuclease, both 3′ and 5′ exonucleases are involved in the degradation of small single-stranded oligonucleotides. Our findings, extending in an unexpected domain the aphorism 'what is true for *E.coli* is true for the elephant', show that at least one indirect target of lithium treatment is remarkably evolutionarily conserved, suggesting that the role of pAp is probably more important than previously suspected.

MATERIALS AND METHODS

Strains, plasmids, growth conditions

E.coli strains were grown at 30°C in MOPS-minimal medium (10) containing 40 µg/ml of amino acids as indicated, K-phosphate at 2 mM, vitamin B1 at 0.0005%, glycerol at 0.4%, glucose or arabinose as indicated. Ampicillin (100 µg/ml) or kanamycin (25 µg/ml) was added for plasmid maintenance or to select for chromosomal marker. CC118 (11) was a gift from Elie Dassa. The plasmid for overexpression of His-tagged Sfn, phyjeR-His was kindly provided by David M. Wilson III and is described in Ref. (12).

Two CysQ deletion mutants were created following the protocol of Yu *et al*. (13). Strain CF10230 was used for mutagenesis. This strain was kindly supplied by Michael Cashel and is a nic+ derivative of DY329 (M. Cashel, unpublished data). The kanamycin resistance cassette was PCR-amplified from FB8PPC::kan, a gift from Francis Biville (F. Biville, unpublished data). Primer UM144, 5′-GGGTATAAGTAAAACAATAAGTTAACACCGCT-CACAGAGACGAGGTGGAGAAgccacgttgtgtctcaaaatctc-3′ (capitalized letters indicate the homology region to the *E.coli* chromosome); and UM146, 5′-TTTAGGTTGGGAGA-AATCAGGATGGCAGAATCAGGAAATACACTGTTTCT-ccgtcccgtcaagtcagcgta-3′ were used for PCR of a fragment used to delete the *cysQ* open reading frame (ORF) completely creating mutant 1. A PCR fragment amplified by UM145: 5′-CTTGCACGGAATGCAGGCGATGCCATTATGCAGG-TCTACGACGGGACGAAAgccacgttgtgtctcaaaatctc-3′ and UM146 was used to create a *cysQ* mutant that leaves the first 24 amino acids intact, designated as mutant 2. This region coding for the N-terminal 24 amino acids was suggested to contain a promoter for *cpdB*, which is transcribed in the opposite direction of *cysQ* by one study (3) but not by another (14). Both mutants behaved identically under all conditions tested here. Mutants were verified by confirmation of the 5′ site of integration into the chromosome by means of PCR using primers UM147, 5′-ACCGTCGCTGCATTCACACT-3′; and UM148, 5′-TGCTTGATGGTCGGAAGAGG-3′ yielding a 618 bp fragment for mutant 1 and a 690 bp fragment for

mutant 2, as well as the 3′ site of integration using primer UM149, 5′-AGACCGATACCAGGATCTTG-3′; and UM150, 5′-CTTATCCATGGCGTGAACAG-3′ yielding a 656 bp fragment for both mutants.

All experiments were performed in accordance with the European regulation requirements concerning the use of Genetically Modified Organisms (level 1 containment, agreement no. 2735).

cysQ was cloned under control of the arabinose-inducible promoter P_{ara} of pBAD18 by replacing the NheI/XhoI fragment coding for Rel*Seq* 1–224 from pUM131 leaving the region coding for the C-terminal his-tag by a PCR fragment containing the ORF for *cysQ*, amplified from MG1655 (15) chromosomal DNA using primer UM133 (5′-GGGGCTAG-CAAGAAGGAGATATACCatgttagatcaagtatgccagcttgcac-3′) and UM131 (5′-GGGGCTCGAGgtaaatagacactctgaacccg-3′) yielding pUM404. Orn was cloned likewise using primer UM143 (5′-GGGGCTAGCAGGAGGAATTCACCatgagtgc-caatgaaaacaacctgatttggat-3′) and UM139 (5′-GAGAGGTC-GACcagcttgataaaatgctcgcggtagt-3′) resulting in pUM408. Chromosomal DNA for use as PCR template was prepared using FTA cards (Whatman).

Purifiction of his-tagged CysQ, Orn and Sfn

CysQ and Orn were purified from 100 ml cultures, of MG1655 carrying the appropriate plasmid, grown at 30°C to an A_{600} between 0.7 and 0.9, and then induced for 3 h with 0.2% arabinose. Cells were harvested and washed once with 50 mM Na-phosphate, 300 mM NaCl before freezing. Frozen pellets were resuspended in 1.5 ml binding buffer [50 mM Na-phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole and 0.4 mM phenylmethylsulfonyl fluoride (PMSF)] and lysed by incubation on ice for 30 min with 130 µg/ml lysozyme followed by sonication. After removal of cell debris by centrifugation at 14 000 r.p.m. for 30 min, proteins were batch purified on 500 µl Ni-agarose (Quiagen) according to the manufacturer's instructions. Wash buffer contained 20 mM imidazole. Elution was done using four aliquots of 500 µl of elution buffer (same as binding buffer, but containing 250 mM imidazole). The most pure fraction was identified by SDS–PAA gel electrophoresis, dialyzed against 50 mM HEPES, pH 7.5 and 100 mM NaCl and kept on ice for storage. For purification of Sfn, the protocol needed to be adjusted due to the lower expression level of this protein. Higher culture volume (400 ml instead of 100 ml) and less Ni-agarose 300 µl instead of 500 µl were used. Induction was done by adding 1 mM isopropyl-β-D-thiogalactopyranoside for 2 h 45 min before harvesting the cells. Binding was done in the presence of 5 mM imidazole instead of 10 mM.

pAp-agarose binding

Cyanogen bromide activated agarose beads (Sigma–Aldrich) were blocked with 0.2 M glycine according to the instructions of the supplier and stored at 4°C in 1M NaCl containing 0.02% sodium azide. pAp-agarose (Sigma–Aldrich) was stored likewise after swelling and washing in H_2O as indicated in the protocol of the manufacturer. Before use, agarose beads were washed three times with at least 10 vol of pAp-agarose buffer [as in (4), 50 mM HEPES, pH 7.5, 10 mM $CaCl_2$ and 50 mM KCl). Cultures of CC118 (200 ml) were grown at 30°C to an

A_{600} between 0.7 and 0.8 in MOPS-minimal medium containing all amino acids but cysteine and methionine and 2% glucose, harvested and washed once in 50 mM Na-phosphate pH 8.0, 300 mM NaCl before freezing. Frozen pellets were resuspended in 2 ml pAp-agarose buffer containing 0.4 mM PMSF and lysed as described above. Debris-free cellular extract was added to 200 µl blocked agarose beads and rotated for 2h at 4°C to clear the extract from proteins binding non-specifically to agarose. Supernatant was divided equally and added to 50 µl of washed pAp-agarose (pAp-binding fraction) or blocked agarose beads (control). After rotation at 4°C for 1 h 30 min, the beads were washed extensively (10 times 800 µl) with pAp-agarose wash buffer (as pAp-agarose buffer but 0.5 M NaCl), and washed once with pAp-agarose buffer to remove excessive salt. Elution was done by adding 75 µl of hot SDS-sample buffer, and incubation at 65°C for 15 min. Beads were removed by centrifugation, and 20 µl of each sample was analyzed by gel electrophoresis on 12% SDS–PAA gels. Bands corresponding to proteins binding specifically to pAp were localized by staining with Bio-Safe colloidal coomassie (Bio-Rad), cut out and digested in-gel by trypsin after reduction and alkylation according to standard protocols and analyzed by nano-HPLC (LC Packing) directly coupled to an ion trap mass spectrometer (ThermoFinnigan LCQ Deca XP) equipped with a nano-electrospray source (LC-MS/MS). Database searches and evaluation of the results was done using the Bioworks 3.1 software. Overall scores were obtained using a multiple threshold filter for individual peptides: 1.5 for one charge, 2 for doubly charged and 2.5 for triply charged ions.

For pAp-binding experiments with human cells, HeLa cells were grown in spinner flasks in DMEM including 7% of fetal bovine serum. Hundred million cells were used to prepare cytoplasmic extract according to Dignam (16).

Orn/Sfn activity assay

Custom-made RNA or DNA oligo 5mers (5′Cy5-CCCCC3′), obtained from Proligo or Sigma-Genosys, respectively, were used as substrate for *in-vitro* activity assays. An aliquot of 30 µl reactions containing 50 mM HEPES, pH 7.5, 5 mM $MnCl_2$, 3 µM substrate and Orn or Sfn as indicated were incubated at 37°C, and reactions were initiated by adding substrate or by adding enzyme. Inhibitors were added as indicated in the text. At intervals, 4.5 µl reaction aliquots were taken and stopped by adding an equal volume of sample buffer (4× TBE, 100 mM DTT, 16% glycerol and 20 mM EDTA) and frozen at −20°C. For analysis of the reaction products, 1.5 or 2.5 µl of samples were applied to PAGE on a 22% SDS–PAA gel containing 2× TBE and run in 2× TBE. Fluorescent RNA oligos were visualized using a Molecular Dynamics STORM 860 in 650 nm long-pass filter mode. Quantification of the data was done by defining the total amount of fluorescence measured in the substrate (5mer) and the reaction products (4mer, 3mer, 2mer, 1mer) for each time point 100% and expressing the amount of each reaction product as fraction of the total.

CysQ activity assay

CysQ activity was tested in 30 µl reactions containing 50 mM HEPES, pH 7.5, 50 mM KCl, 200 mM NaCl, 2 mM $MgCl_2$ and 5.5 mM pAp at 37°C, reactions were started by the addition of 0.5 µg CysQ. Inhibitors were added as indicated. At intervals,

4.5 µl samples were mixed with 0.5 µl of 100 mM EDTA, and resolved by polyethyleneimine (PEI) thin-layer chromatography with 0.8 M LiCl as solvent. Authentic AMP and pAp were used as migration standards. Accumulation of reaction products was estimated after visualization by UV.

RESULTS

Identification of *E.coli* proteins binding to pAp, CysQ and Orn

As a starting point to explore the regulatory function of pAp, we chose the identification of proteins interacting with this nucleotide, namely pAp-affinity chromatography. We chose to do initial experiments on extracts of *E.coli* for two reasons: (i) we were interested to learn if potential regulatory mechanisms involving pAp could be evolutionary conserved and (ii) experiments are easier to set up using bacterial extracts. We used *E.coli* CC118, a strain harboring a deletion, for alkaline phosphatase in order to prevent degradation of the affinity substrate. $CaCl_2$ was included in the buffer for binding in the anticipation that it can prevent degradation of pAp by pAp-phosphatase as pAp-phosphatases were shown to be sensitive to $CaCl_2$ (17–19). In addition, these conditions had been proven useful for the purification of the human pAp-phosphatase, BPNT1(4).

Three major protein bands became visible in the fraction of protein binding specifically to pAp (Figure 1). Analysis of these bands by LC-MS/MS or direct sequencing revealed CysQ (SwissProt, P22255) for band number 1, HisIE (SwissProt, P06989) for band number 2, and oligoribonuclease, Orn (SwissProt, P39287) for band number 3.

CysQ was identified with an overall score of 860 represented by five distinct peptides, covering 22.3% of the total mass of the protein. Orn was identified with an overall score of 768, eight peptides covering 42% of the total protein mass
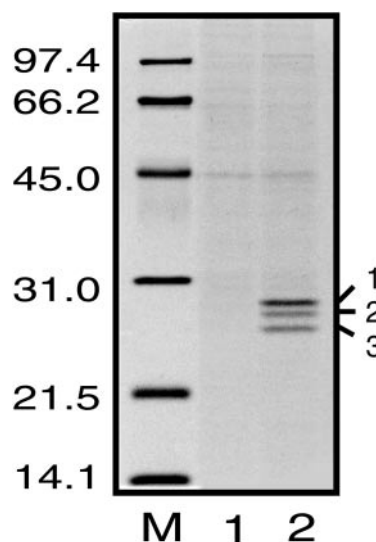

Figure 1. pAp-binding proteins from *E.coli*. Shown is colloidal coomassie stained SDS–PAA gel separating the pAp-binding fraction of *E.coli* extract. M, marker; lane 1, fraction binding to blocked agarose beads (control); lane 2, pAp-binding fraction.

were identified. HisIE was identified by sequencing of the following tryptic peptide, VGEEGVETAL.

The experiments described in the next part were intended to characterize two of the proteins binding to pAp, CysQ and Orn and in particular the consequences of their interaction with pAp. The characterization of HisIE and the potential biological consequences of the interaction of this protein with pAp are not objects of this study.

CysQ is a main target for lithium toxicity in *E.coli*

Like its homolog in yeast, Hal2 (20) and the mammalian counterparts (BPNT1) (4,19), CysQ was shown to degrade pAp to AMP (4). The identification of CysQ as a protein binding to pAp was therefore expected. Recovery of this protein shows that cross-linking to the matrix does not interfere with major features of pAp as a substrate for specific interactions.

CysQ carries a conserved sequence motif, $D(X)_n$ $EE(X)_nDP(I/L)D(S/G/A)T(X)_nWD(X)_{11}GG$ required for metal ion coordination that places it into a larger family of magnesium-dependent phosphomonoesterases with otherwise low overall sequence similarity but a conserved 3D core structure (6). Members of this family are predicted to be highly sensitive to lithium, but this requires experimental substantiation. To our knowledge, sensitivity of CysQ to lithium and other salts was not tested before. We therefore decided to test lithium sensitivity of CysQ using a his-tagged protein that was purified to homogeneity. The his-tagged CysQ was shown to be biologically functional by demonstrating its ability to complement the phenotype of a *cysQ* deletion, namely a growth defect in the absence of cysteine, when overexpressed under the control of the arabinose-inducible promoter P_{BAD} in pBAD18 (Table 2).

As expected, CysQ-catalyzed pAp hydrolysis requires Mg^{2+} (data not shown). Sensitivity to calcium was reported for pAp phosphatases. For this reason $CaCl_2$ was included in the test. Figure 2 shows complete inhibition of CysQ in the presence of either 1 mM LiCl or 2 mM $CaCl_2$. Reactions were performed in the presence of 200 mM NaCl. Under these conditions CysQ activity was higher than in the absence of NaCl. Thus, sodium is not inhibitory at physiological concentrations and does not interfere with lithium. Importantly, this places CysQ in the class of sodium tolerant pAp phosphatases, together with mammalian rat BPNT1 (19) and human BPNT1 (7) and two homologous proteins in *Arabidopsis*, Sal1 and Sal2 (18), and contrasts it with the yeast proteins Hal2 (8) and Tol1 (21) and the third pAp-phosphatase in Arabidopsis, AHL (18).

As mentioned in the introduction, recent data show that pAp-phosphatase Hal2p is a main target of lithium toxicity in yeast. The observed sensitivity to lithium raises the possibility that similarly, CysQ might be the main target of lithium toxicity in *E.coli*. Evolutionary conservation of targets of sensitivity to lithium between different domains of life is intriguing in light of the existence of conservation of regulatory pathways in general and the interest in the molecular mechanisms of lithium action in particular. To test this hypothesis, the wild-type strain MG1655 was grown in the presence of sulfate as sole sulfur source. Under these conditions, cysteine needs to be synthesized through the assimilation of sulfate, and pAp is produced as a by-product. Adding LiCl should inhibit pAp-phosphatase and lead to an accumulation of pAp. A considerable inhibitory effect of lithium on cell growth was observed. Obvious differences in colony size were detected already after plating cells in the presence of 50 mM LiCl (data not shown). Severe growth inhibition was observed at 100 mM LiCl (Figure 3, upper panel). Importantly, this growth defect could be almost completely rescued by overexpression of CysQ (Figure 3, lower panel). Similar experiments were done in liquid medium. Addition of increasing amounts of LiCl showed increasing inhibition of growth (Table 1). As observed when growing on plates, overexpression of CysQ rescues the growth defect completely. It can therefore be concluded that CysQ is a main target for Li-toxicity under the conditions used. Thus, it is very probable that as in yeast additional direct targets for lithium toxicity do not exist. For further confirmation of this conclusion it would be interesting to test if additional suppressors of lithium toxicity can be identified from a multicopy library.

Orn is a potential downstream target of pAp

As lithium-toxicity is caused mainly by accumulation of pAp, the question arises what are the downstream targets of pAp accumulation. We expected that potential targets should be found among the proteins binding to pAp, and identified Orn as one of these proteins.

Orn is an essential protein in *E.coli* involved in mRNA degradation. It has 3′–5′ exoribonuclease activity selectively

Table 1. Rescue of Lithium toxicity by overexpression of CysQ but not Orn

Plasmid	Doubling time (min) for growth in the presence of		
	0 mM LiCl	100 mM LiCl	200 mM LiCl
pBAD18 (vector)	84	101	114
pUM404 (CysQ-His)	87	84	85
pUM408 (Orn-His)	92	104	120

Strain CF10230 carrying plasmids as indicated was grown in MOPS-minimal medium containing all amino acids but cysteine, glycerol at 0.4% and arabinose at 0.02% for induction of P_{ara}. LiCl was added as indicated at an OD_{600} of ~0.1, and doubling times were measured thereafter.
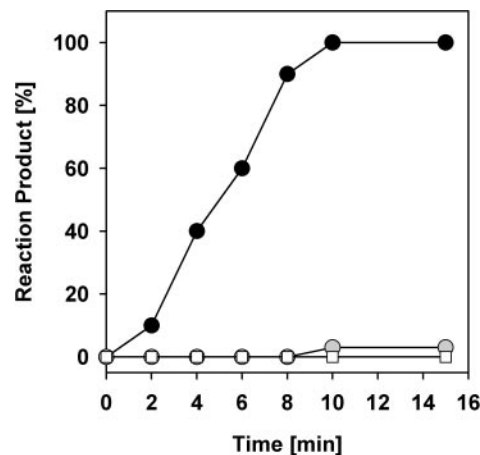

Figure 2. Inhibition of recombinant CysQ by Lithium (1 mM) and Calcium (2 mM). Reactions contained 5.5 mM pAp and 0.5 µg of CysQ. Closed circles, no inhibitor; gray circles, LiCl; open squares, $CaCl_2$.
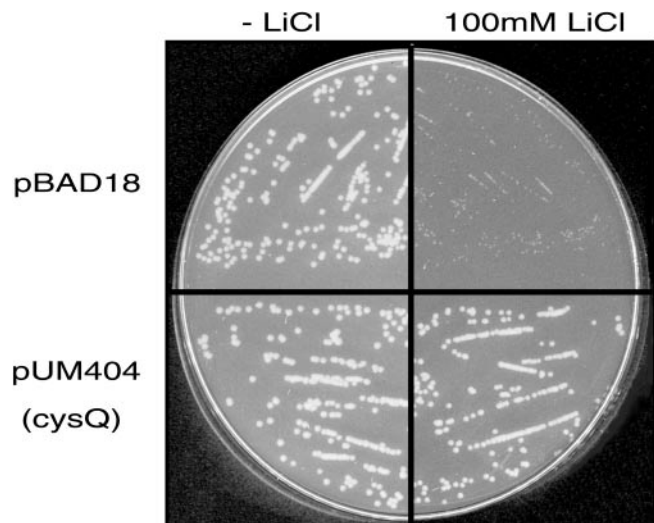
Figure 3. Lithium induced growth inhibition in *E.coli* MG1655 containing pBAD18 (vector control) or pUM404 (arabinose-inducible *cysQ*). Strains were grown on MOPS-minimal plates containing all amino acids but cysteine and methionine, 0.4% glycerol and 0.0002% arabinose.

active on short ribonucleotides (22–25). Given the strong affinity of Orn for pAp, we asked first if pAp is a possible substrate for Orn. Reactions were done as described for Figure 2 for CysQ except that twice the amount of enzyme was used (1 μg instead of 0.5 μg). CysQ served as positive control. Under these conditions, all pAp was converted into AMP after 5 min by CysQ. Orn was tested in the presence of Mg2+ and Mn2+ at 2 mM. No conversion of pAp into AMP was observed during 30 min (data not shown). pAp is therefore not a substrate for Orn under the conditions tested.

Next we wanted to investigate if pAp has an effect on Orn activity *in vitro*. Orn was shown to be most active on single-stranded RNA oligonucleotides of ≤5 nt in length (23,24). RNA oligo 5mers (5′-CCCCC-3′) labeled at their 5′-phosphate end with the sulfoindocyanine succinimidyl ester cyanine 5 (Cy5) were therefore used as substrate for *in vitro* assays with purified recombinant Orn. Separating reaction products on a 22% SDS–PAA gel, we observed a reverse migration phenomenon. This effect can be accounted for by the fact that cyanine dyes have a lower net negative charge than nucleic acids; thus, removing nucleotides will reduce the charge relative to the mass of the oligonucleotide and cause it to shift up instead of down.

The sequential appearance of reaction products of different chain length (Figure 4) is in agreement with previous data (24) and the proposed processive reaction mechanism of oligoribonuclease. We tested Orn activity in the presence of different concentrations of pAp (10, 20 and 50 μM). Increasing inhibition can be seen with increasing concentrations of pAp. We chose to present here the kinetics of oligo-RNA degradation in the presence of 20 μM pAp together with the control lacking the inhibitor as this concentration of pAp causes a strong and easily observable effect.

Preliminary results regarding salt sensitivity of Orn show no sensitivity to LiCl up to 300 mM, and slight inhibition by NaCl and CaCl$_2$ starting from concentrations exceeding

100 mM with NaCl being more inhibitory than CaCl$_2$ (data not shown).

Overexpression of *orn* cannot rescue the *cysQ* mutant phenotype or the lithium effect on growth

Given the observed strong inhibition of Orn by pAp together with the fact that Orn is essential in *E.coli*, we asked if the *cysQ* mutant phenotype could be rescued by overexpressing Orn. For this purpose growth tests were performed on MOPS-minimal plates or in liquid medium. As shown in Table 2, overexpression of Orn could not rescue the growth inhibition seen in both *cysQ* mutants grown in the absence of Cysteine. Also, Orn overexpression could not rescue the growth inhibitory effect of lithium in the wild-type strain (Tables 1 and 2). As mentioned earlier, both effects can however be rescued by *cysQ* overexpression. This raises the possibility that additional downstream targets of pAp accumulation exist in *E.coli*. Alternative explanations are discussed below.

Identification of pAp-binding proteins in human cells

The possibility that pAp accumulation caused by lithium-inhibition of pAp-phosphatase may explain some of the effects of lithium, either toxic or therapeutic, prompted us to extend our search for pAp-binding proteins to human cells. Cytoplasmic extracts of HeLa cells were dialyzed against pAp-agarose buffer and concentrated (10-fold) before binding experiments were performed in a similar way as described for bacterial extracts. Figure 5 shows the presence of three major proteins that bind specifically to pAp. Identification of band number 2 by LC/MS-MS revealed nucleoside diphosphate kinase (NDK) with an overall score of 280, 6 peptides covering 35% of the total mass: DRPFFAGLVK, FMQASEDLLK, GDFCIQVGR, GLVEIIK, NIIHCSDSVESAEK and TFIAIKPDGVQR.

Band number 3 was also identified as NDK with an overall score of 158, and 4 peptides covering 16% of the total mass: TFIAIKPDGVQR, PDGVQR, which is contained in peptide 1 and the result of a more complete trypsin digestion, GDFCIQVGR and GLVEIIK.

NDK exists in different isoforms and different functions have been reported for individual isoforms (Discussion). The peptides identified for bands number 2 and number 3 do not allow distinguishing unequivocally between these isoforms, differences in the molecular mass weight however point to NDK A (SwissProt, P15531) for band number 3 with a predicted molecular mass weight of 17.1 kDa and a transcriptional variant of the same gene (Uniprot, Q86XQ2) (26) for band number 2 (19.6 kDa).

NDK was reported to be inhibited by 3′-phosphorylated nucleotides *in vitro*, in particular by pAp and PAPS (27). Therefore, further validation of this finding was not pursued.

A downstream target of pAp accumulation is conserved between *E.coli* and humans: Sfn

Identification of band number 1 revealed small fragment nuclease (Sfn) with an overall score of 338, and 7 peptides covering 31% of the total mass. Sfn is the human homolog of *E.coli* Orn and shares a high degree of similarity (50%) with its bacterial counterpart (28). Sfn and Orn belong to a large family of 3′–5′ exonucleases that are characterized by three ancient
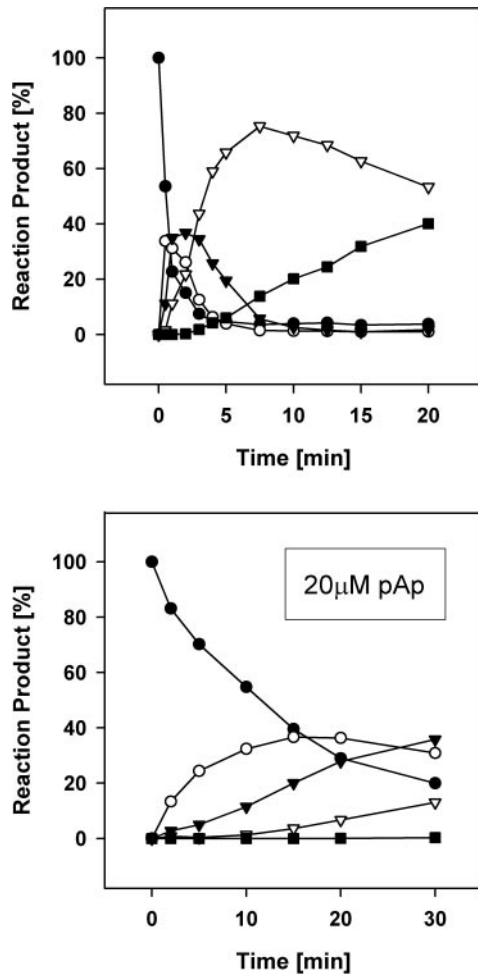
Figure 4. Orn is sensitive to micromolar amounts of pAp. Shown is the quantification of the distribution of reaction products that were separated on 22% PAA gels. Reactions included 0.07 µg Orn and 3 µM of RNA oligo 5mer (5′Cy5-CCCCC3′). Reactions were started by the addition of substrate. pAp (20 µM) was present where indicated. Closed circle, 5mer; open circle, 4mer; closed triangle, 3mer; open triangle, 2mer; square, 1mer.

conserved exonuclease motifs (exo I, II and III) (29,30). As does Orn, Sfn specifically degrades short oligoribonucleotides of ≤5 nt in length. In addition, Sfn was shown to be active on small DNA oligos (12). A role of Sfn in cellular nucleotide recycling was therefore suggested. We purified his-tagged Sfn in order to assess its sensitivity to pAp *in vitro*. As compared with Orn, 7-fold more Sfn was necessary to obtain comparable activity. The reason for this might lie in the fact that the human enzyme was expressed in *E.coli*, which differ from mammalian cells in folding and posttranslational modification mechanisms. Inhibition was tested at two concentrations of the inhibitor, 20 and 50 µM. As shown in Figure 6, significant inhibition could be seen in the presence of 20 µM pAp, and increasing inhibition was observed at 50 µM.

Initial experiments to test sensitivity of Sfn-catalyzed DNA oligo degradation showed that pAp inhibits this activity of Sfn as well (data not shown).

The recognition of Sfn as a pAp-regulated enzyme demonstrates that at least one target of pAp accumulation is conserved between *E.coli* and humans.

Orn is active on DNA oligos

Both Orn and Sfn belong to the 3′–5′ exonuclease superfamily, which includes proofreading domains of all known DNA-dependent DNA polymerases (28). Therefore Orn might be able to degrade short DNA oligos as well, a possibility that was never tested when this enzyme was characterized as a ribonuclease, since only double-stranded T7 DNA was used as a DNA substrate (23). Figure 7 shows Orn activity on Cy5-labeled oligo-DNA. 10-fold higher enzyme concentrations were necessary to obtain degradation of DNA oligos as compared with RNA oligos. Similar preferential degradation of RNA over DNA had been shown for Sfn (12).

DISCUSSION

The purpose of this study was to explore the regulatory function of the nucleotide, pAp that is generated in both prokaryotes and eukaryotes in the process of sulfur assimilation. In addition to our interest in possible parallels of regulatory pathways between these two domains of life, pAp is of particular interest for its possible involvement in the molecular effect of lithium in the treatment of bipolar disorder. The beneficial effect of lithium on patients with bipolar disorder was described for the first time >50 years ago (31). Efforts to identify targets of lithium concentrated to a large extent on its action on glycogen synthase kinase (GSK)-3 (32,33) and inositol monophosphatase (34,35). Those and other hypotheses are reviewed (36,37). We focus here on lithium's action on members of a structurally conserved family of metal dependent phosphomonoesterases that include inositol monophosphatase (6) and more particular on pAp-phosphatase, as the K_i for lithium-inhibition of the human homolog with 0.3 mM (7) is the lowest K_i described for lithium-inhibited enzymes. pAp accumulation due to the inhibition of pAp-phosphatase could explain some of the effect of lithium treatment. Our search for regulatory targets of pAp was carried out in *E.coli* and in human cells and started by the identification of proteins binding to pAp followed by the attempt to understand the biological consequences of the interaction of pAp with some of these proteins. Among the proteins binding to pAp, we expected to find proteins that have pAp as a substrate or proteins that are regulated by pAp. In CysQ from *E.coli*, we found a protein that uses pAp as substrate and cleaves the 3′ phosphate, leaving AMP as a metabolite directly recycled in metabolism by adenylate kinase. We demonstrate that CysQ, like other members of a structurally conserved protein family, is highly sensitive to lithium and calcium *in vitro*. Furthermore, CysQ is not inhibited by physiological concentrations of sodium like mammalian pAp-phosphatases, rat BPNT1 (19) and human BPNT1 (SwissProt, O95861) (7) and two out of three homologous proteins in *Arabidopsis* (Sal1 and Sal2) (18) and unlike the yeast proteins Hal2 (P32179) (8) and Tol1 (21) and a third homolog form *Arabidopsis*, AHL (18).

Interestingly, CysQ is a main target of lithium-toxicity in *E.coli* as overexpression of this protein can rescue the growth defect observed when bacterial cells are treated with LiCl. A similar effect was observed in yeast (9). These authors demonstrated a direct correlation between growth inhibition and pAp levels when levels were manipulated either by genetics or pharmacological adjustment. The evolutionary conservation

Table 2. Overexpression of *orn* cannot rescue the *cysQ* Mutant phenotype or the toxicity effect of Lithium

| Strain | *cysQ* allele | Plasmid | Growth on MOPS Minimal | | |
			+Cys	−Cys	−Cys, +LiCl
UM304	Wild type	pBAD18 (vector)	+	+	−
UM305	Wild type	pUM404 (P_{ara}:*cysQ*-His)	+	+	+
UM306	Wild type	pUM408 (P_{ara}:*orn*-His)	+	+	−
UM307	Mutant 1	pBAD18 (vector)	+	−	−
UM308	Mutant 1	pUM404 (P_{ara}:*cysQ*-His)	+	+	+
UM309	Mutant 1	pUM408 (P_{ara}:*orn*-His)	+	−	−
UM310	Mutant 2	pBAD18 (vector)	+	−	−
UM311	Mutant 2	pUM404 (P_{ara}:*cysQ*-His)	+	+	+
UM312	Mutant 2	pUM408 (P_{ara}:*orn*-His)	+	−	−

Bacteria were grown on plates or in liquid culture of MOPS-minimal medium containing all amino acids or lacking cysteine. LiCl was present at 100 mM in plates and at 100 or 200 mM in liquid culture. Arabinose was present at 0.02% for induction of P_{ara}. Plus indicates normal growth, and minus indicates inhibited growth.
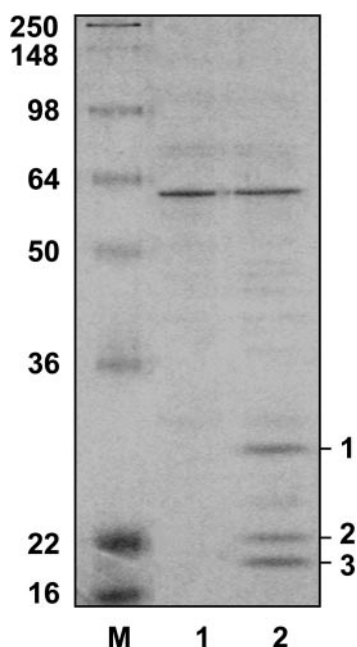

Figure 5. pAp-binding proteins from humans. Shown is colloidal coomassie stained SDS–PAA gel separating the pAp-binding fraction of HeLa cytoplasmic extract. M, marker; lane 1, control; lane 2, pAp-binding fraction.

of targets of sensitivity to lithium between different domains of life is intriguing as it points to conservation of regulatory pathways.

As an example for proteins regulated by pAp, we found oligoribonuclease, Orn from *E.coli*. We show that this protein is inhibited by micromolar amounts of pAp *in vitro*. Orn was tested previously for inhibition by 5′- or 3′-mononucleotides (24). That work found that 3′ ribonucleotides did not have an effect on Orn activity, whereas 1 mM of 5′ ribonucleotides (a concentration that is much higher than what we used in our assays for pAp) inhibited Orn activity to various extents, depending upon the particular nucleotide: GMP and AMP were more inhibitory than UMP and CMP. 5′-Ribonucleotides are the end products of Orn reaction and they are therefore expected to contribute to modulation of Orn activity. The levels of mononucleotides in the cell are estimated to be lower than 100 µM (38). The observed toxicity of LiCl treatment in *E.coli* suggests that, as in the case of yeast (8,9), pAp accumulates to considerably higher amounts than ribomononucleotides.

Remarkably, when using HeLa cell extract, we found the human homolog of Orn, Sfn to bind to pAp-agarose. *In vitro* assays with his-tagged Sfn show that like Orn, Sfn is highly sensitive to pAp. Orn and Sfn share a distinct substrate preference for oligoribonucleotides shorter than 5 nt *in vitro*. In *E.coli*, Orn is the only exoribonuclease that is able to degrade small oligoribonucleotides (39), and short oligoribonucleotides (2–5 nt) were shown to accumulate under circumstances of a conditional knockout (22). Orn is encoded by an essential gene, which is unusual for *E.coli* exoribonucleases. Ghosh and Deutscher (22) consider three different explanations why the absence of Orn activity leads to cessation of growth: (i) depletion of the cells' mononucleotide pool, (ii) inhibition of certain enzymes of essential metabolic processes by accumulated oligoribonucleotides, or (iii) a yet unknown function of Orn that is essential.

The human Orn homolog (Sfn) was shown to degrade small DNA oligos in addition to oligoribonucleotides (12). These short DNA oligos could emerge from DNA repair and recombination and indeed it was shown that Sfn plays a role in resistance to UV-C (40). Both Orn and Sfn belong to the 3′–5′ exonuclease superfamily, which includes proofreading domains of all known DNA-dependent DNA polymerases (28): we therefore decided to test the possibility that Orn might be able to degrade short DNA oligos as well. We show that, as for Sfn (12), DNA directed exonuclease activity of Orn exists but requires higher enzyme concentrations than RNA directed activity. Thus, as for Sfn, Orn could play a possible role in DNA repair. Owing to the conservation of the active center of exonucleolytic activity within the superfamily (29,30) one might wonder if the pAp-exerted inhibition extends to other members as well. Notably, some of the members of this superfamily were linked to human diseases like Werner syndrome and polymyositis-scleroderma overlap syndrome 100 kDa autoantigen (41,42).

It is noteworthy that the effect reported here of pAp on RNA degradation is not the first example for the connection between sulfur and RNA metabolism. In fact, inhibitory effects of pAp on exonucleases Xrnp1 and Rat1p, two enzymes homologously conserved throughout eukaryotes [reviewed in (43)], were reported before (5). Xrnp1 and Rat1p are required for correct 5′ processing of 5.8S RNA and snoRNA, degradation of pre-RNA spacer fragments and mRNA turnover (5). Stabilization of mRNA and subsequent changes in expression patterns as well as additional changes in RNA metabolism due to pAp-mediated inhibition of Xrn1p and Rat1p were
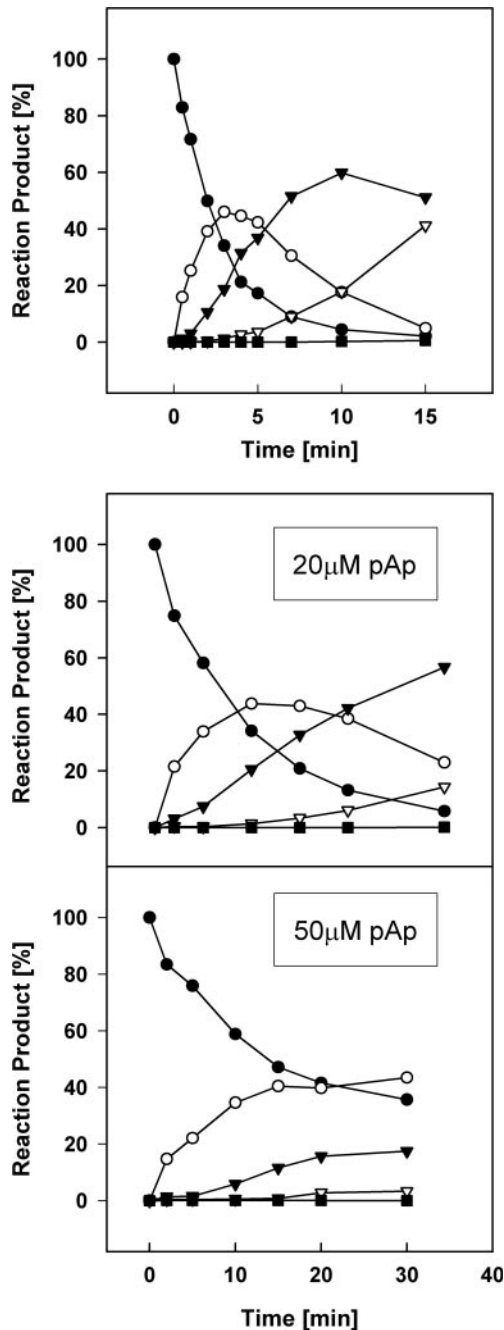
Figure 6. Sensitivity of Sfn to pAp. Shown is the quantification of the distribution of reaction products that were separated on 22% PAA gels. Reactions included 0.5 µg Sfn and and 3 µM of RNA oligo 5mer (5′Cy5-CCCCC3′). Reactions were started by the addition of enzyme. pAp (20 or 50 µM) was present where indicated. Closed circle, 5mer; open circle, 4mer; closed triangle, 3mer; open triangle, 2mer; square, 1mer.

Figure 7. Orn is active on DNA oligos. Reactions contained 3 µM of DNA oligo 5mer (5′Cy5-CCCCC3′) and were started by the addition of 1 µg of Orn. Dash labels a control lacking enzyme incubated for 20 min. Samples were taken as indicated. The DNA monomer cannot enter the gel. The different gel mobility as compared with RNA is caused by the one missing charge of DNA. Varying the pH of the electrophoresis buffer could not change this effect.

proposed to account for developmental effects observed under lithium treatment. More recent results on the Xrn1p homolog from Arabidopsis point to specific mRNA targets for this enzyme and to its involvement in miRNA mediated cleavage (44).

Xrnp1 and Rat1p specificities are directed oppositely from oligoribonucleases, namely from 5′ to 3′. To our knowledge, our study reports for the first time an exonuclease directed from 3′ to 5′ to be inhibited by pAp. In fact in yeast, 3′–5′ directed
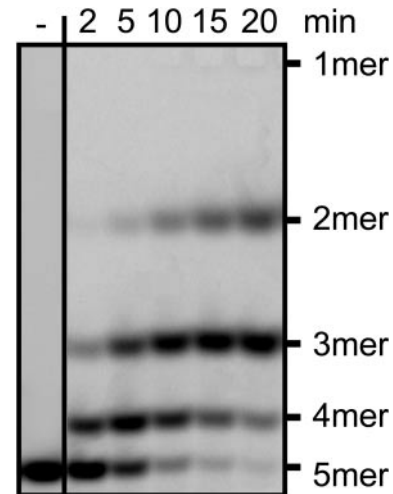
degradation of mRNA was shown to be unaffected by pAp (45). The recurring finding of a connection between pAp and RNA metabolism is of interest in light of the effect of RNA stability on gene expression. In the case of Xrn1P, complex changes of expression pattern were shown in mutants lacking this protein (46). Similarly, pAp-mediated inhibition of oligoribonucleases might have effects on gene expression. Ubiquitous expression of Sfn points to an important role of this protein in mammals (12). In the absence of a complete understanding of the role that oligoribonucleases and in particular Sfn play, we can only speculate about the possible consequences of their inhibition. In this respect, it is interesting that Orn activity was shown to display some degree of sequence specificity (24), and therefore, its function could be targeted to a more specific set of short oligoribonucleotides. The role of small RNAs, microRNAs and siRNAs in higher organisms as well as small non-coding RNAs in bacteria in the regulation of gene expression is increasingly recognized and has seen enormous advances in the last decade [for reviews see (47–52)]. It is conceivable that short oligos could interfere with the metabolism of those small RNAs by means of hybridization and thereby contribute to changes in gene expression. A recent work investigating the requirements for functional miRNAs (53) shows that 5′homology of 4–5 nt between miRNA and its target site is sufficient to function as so called 'seed' if it is accompanied by an extensive 3′ homology region following a central bulge. In addition, 5′ seeds of 8 nt homology are sufficient even in the absence of 3′ homology. RNA oligo 2–5mers could therefore potentially compete with the pairing between miRNA target and seed region of the miRNA. In addition, the ability of short oligos to enter the transcription bubble could be an alternative route of interference with gene expression.

Essentiality of Orn in *E.coli* raised the possibility that this downstream target of pAp could rescue the effects of pAp accumulation observed in a *cysQ* mutant or in the wild type under conditions of lithium treatment. We found this however not to be the case. Although it is possible that due to the high

sensitivity of Orn to pAp, overexpression might not be able to overcome pAp-inhibition; an alternative explanation is the existence of additional targets of pAp. Indeed, our search for pAp targets is likely to be not exhaustive and restricted to the finding of proteins with higher expression levels.

The second human enzyme we found to bind to pAp is NDK. The inhibition of NDK by 3′-phosphorylated nucleotides, in particular pAp and PAPS, was already reported (27); however, our approach provides an independent confirmation of the physiological significance of this inhibition. In addition to maintenance of (d)NTP levels, NDKs were suggested to have other important metabolic functions in processes such as signal transduction (54) and developmental control (55).

In humans there exist eight different and widely expressed NDKs, some of which play a critical role in development (56–59). NDK A and NDK B are the most abundant and closely related (88% identity) isoforms. The two NDK homologs were shown to act as suppressors of tumor metastasis but also to function in tumorogenesis, as reviewed in Ref. (60,61). NDK B binds specifically to the promoter of the c-myc oncogene and activates its transcription (62). This activation involves structural rearrangements through cleavage of a nuclease hypersensitive region (63). Of interest for the pAp-inhibitory effect is the fact that the residue required for this cleavage reaction, Lys12, lies in the catalytic pocket involved in the NDP kinase phosphorylation reaction (64). pAp binds to NDK at the same site as the physiological substrates (d)NTP or (d)NDP without disturbing the conformation of the active site in an inverted position where the 3′ phosphate occupies the space the γ-phosphate of ATP would reside in (27) close to the catalytic histidine. ATP but not ADP was shown to inhibit the cleavage reaction with 50% inhibition at 0.5 mM (65). Considering the fact that it is specifically the γ-phosphate and the adenine base that inhibits the cleavage and not ATP hydrolysis or phosphorylation of the catalytic histidine, we predict that pAp will inhibit not only NDP kinase activity but also DNA cleavage required for transcription activation of c-myc as well as repair (66). This is the more likely as pAp makes polar interactions with Lys12 (27) required for cleavage. A most interesting feature of this remarkably conserved role is that it links sulfur metabolism to completely unexpected targets. It is therefore of importance to make an exhaustive analysis of pAp targets, as well as of their evolution.

The knowledge of pAp targets can improve the molecular understanding of the multifarious therapeutical and toxic effects of lithium and might lead to more directed pharmacological approaches. Our findings suggest the existence of an additional network of proteins, controlled by pAp levels, that contributes significantly in the mechanisms of lithium action and is independent from the better explored GSK-3, and protein kinase C networks (66).

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