Mn$^{2+}$ Is a Native Metal Ion Activator for Bacteriophage λ Protein Phosphatase†

Tiffany A. Reiter,* Nicholas J. Reiter, and Frank Rusnak‡

Section of Hematology Research and the Department of Biochemistry and Molecular Biology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Received June 18, 2002; Revised Manuscript Received September 13, 2002

ABSTRACT: Bacteriophage λ protein phosphatase (λPP) is a member of a large family of metal-containing phosphoesterases, including purple acid phosphatase, protein serine/threonine phosphatases, 5′-nucleotidase, and DNA repair enzymes such as Mre11. λPP can be activated several-fold by various divalent metal ions, with Mn$^{2+}$ and Ni$^{2+}$ providing the most significant activation. Despite the extensive characterization of purified λPP in vitro, little is known about the identity and stoichiometry of metal ions used by λPP in vivo. In this report, we describe the use of metal analysis, activity measurements, and whole cell EPR spectroscopy to investigate in vivo metal binding and activation of λPP. Escherichia coli cells overexpressing λPP show a 22.5-fold increase in intracellular Mn concentration and less dramatic changes in the intracellular concentration of other biologically relevant metal ions compared to control cells that do not express λPP. Phosphatase activity assessed using para-nitrophenylphosphate as substrate is increased 850-fold in cells overexpressing λPP, indicating the presence of metal-activated enzyme in cell lysate. EPR spectra of intact cells overexpressing λPP exhibit resonances previously attributed to mononuclear Mn$^{2+}$ and dinuclear [(Mn$^{2+}$)$_2$] species bound to λPP. Spin quantitation of EPR spectra of intact E. coli cells overexpressing λPP indicates the presence of approximately 40 µM mononuclear Mn$^{2+}$-λPP and 60 µM [(Mn$^{2+}$)$_2$]-λPP. The data suggest that overexpression of λPP results in a mixture of apo-, mononuclear-Mn$^{2+}$, and dinuclear-[(Mn$^{2+}$)$_2$] metalloisoforms and that Mn$^{2+}$ is a physiologically relevant activating metal ion in E. coli.

The bacteriophage λ protein phosphatase (λPP)$^1$ was originally identified and characterized as a phosphatase by Cohen et al. on the basis of significant amino acid sequence homology with mammalian protein phosphatases (PP) 1 and 2A (9, 10). One hundred fifteen residues of the N-terminal sequence of λPP have 35% sequence identity to the N-terminal sequences of protein phosphatases 1 and 2A (PP1 and PP2A, respectively) (9, 10). PP1, PP2A, and λPP belong to a large family of metallophosphoesterases, which includes bacterial/cyanobacterial (11), archaean (12), fungal (13–15), protist (16), plant (17, 18), and animal (19) protein phosphatases, Mre11 nuclease (7), 5′-nucleotidase (6), and purple acid phosphatase (20–24). The enzymes in this family share a common phosphoesterase motif, DXXH(X)$_3$GDXXD(X)$_n$G-NH$_2$/E (25–27). The amino acids highlighted in bold are situated in loops within a common secondary structural motif, the $\beta$α$\beta_1$α$\beta$-fold, and are metal ligands to an active site dinuclear metal center$^2$ (1–9).

Each phosphatase in the family appears to have different metal ion requirements. Various metalloisoforms of purple acid phosphatase have been isolated, including Fe$^{2+}$/Fe$^{3+}$/Zn, and Fe$^{2+}$/Mn forms (20–24). Both PP1 and PP2A are activated in vitro by Mn$^{2+}$, Co$^{2+}$, and Fe$^{2+}$/ascorbate, but the identity of the native metal ions is not yet resolved (28–32). Calcineurin (PP2B), an Fe$^{2+}$/Zn enzyme activated by Ca$^{2+}$/calmodulin (19, 33, 34), can also be activated in vitro by Mn$^{2+}$ and Ni$^{2+}$, but there is no evidence that Mn$^{2+}$ is an intrinsic metal activator (33, 35–41). λPP and other bacterial phosphatases can be stimulated severalfold by divalent metals, with Mn$^{2+}$ and Ni$^{2+}$ providing the most significant activation (27, 42). The ability of purified λPP to bind a dinuclear [(Mn$^{2+}$)$_2$] cofactor has been confirmed by EPR.

$^1$This work was supported by a grant from the National Institutes of Health, GM46865.

$^2$Corresponding author. Address: 665 Huntington Ave. 1-512, Boston, MA 02115. Tel: (617) 432-2501. Fax: (617) 432-0377. E-mail: treiter@hsph.harvard.edu.

$^4$Deceased.

†Abbreviations: BSA, bovine serum albumin; EPR, electron paramagnetic resonance; λPP, bacteriophage lambda protein phosphatase; λPpT7, bacteriophage lambda protein phosphatase T7-promoter based protein expression vector; ICP-ES, inductively coupled plasma emission spectroscopy; IPTG, isopropyl $\beta$-D-1-thiogalactospyranoside; PMSF, phenylmethylsulfonyl fluoride; $ho$NPP, para-nitrophe- nyl phosphate; PP, protein phosphatase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B (calcineurin); PrPA, protein phosphatase A.

Four variations of the metallophosphatase active site have been classified by the Structural Classification of Proteins (SCOP) database (http://scop.mrc-lmb.cam.ac.uk/scop/). The hallmark is a dinuclear metal center with separation between metal ions from 3.1 to 3.4 Å (1–9).

Of the two metal ions, designated M1 and M2, the M2 site is most highly conserved. In all cases, the ligands to the M2 site are a carboxamide ligand from an asparagine group, two histidine imidazole ligands, and a carboxylate group from a conserved aspartate that bridges M1 and M2 ions, and an additional bridging oxygen ligand usually from solvent. The coordination environment about M1 differs for the four classes: In the protein serine/threonine phosphatases (λPP, PP1, calcineurin A), besides the bridging ligand atoms, the M1 ion is coordinated by a histidine imidazole group and an aspartate carboxylate (1–5). These, plus an additional glutamine ligand, are observed coordinated to the M1 ligand in E. coli 5′-nucleotidase (6), whereas an additional histidine is found in the DNA repair enzyme Mre11 from P. furiosus (7). In the purple acid phosphatases, a tyrosine residue substitutes for the histidine, and a histidine replaces a solvent molecule, for a net tyrosine for solvent substitution in the coordination sphere of the M1 site (8).
Mn$^{2+}$ is a native metal for Bacteriophage $\lambda$PP

Spectroscopy (43, 44) and X-ray crystallography (1). Nevertheless, several other divalent metal ions are suitable activators. Extensive characterization of purified $\lambda$PP has been performed, but little is known about the identity and stoichiometry of metal ions utilized in vivo.

In this report, whole cell EPR spectroscopy, metal analysis, and activity measurements are utilized to investigate metal binding and activation of $\lambda$PP in vivo. The data indicate that $\lambda$PP preferentially binds and is activated by Mn$^{2+}$ when overexpressed in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Protein Expression.** Competent cells of the *E. coli* strain BL-21 Star (DE3)pLysS (Invitrogen) were transfected with one of two plasmids: $\lambda$PPpT77, which contains the $\lambda$PP gene upstream of the T7 polymerase promoter and is used to overexpress $\lambda$PP, or pT7-7, the parent vector without the gene for $\lambda$PP (45). Cells were grown in LB media or M9 minimal media with ampicillin (0.1 mg/mL). For LB media, cultures were grown with or without metal supplementation consisting of 504 $\mu$M FeCl$_3$, 25.6 $\mu$M CaCl$_2$, 20.8 $\mu$M H$_3$BO$_3$, 4.0 $\mu$M MnCl$_2$, 1.52 $\mu$M CoCl$_2$, 49.8 $\mu$M ZnCl$_2$, 50.0 $\mu$M Na$_2$MoO$_4$, and 4.0 $\mu$M NiCl$_2$ (46). For cells grown in M9 minimal medium (per liter: 6 g Na$_2$HPO$_4$, 3 g KH$_2$PO$_4$, 1 g NH$_4$Cl, 0.5 g NaCl, 0.12 g MgSO$_4$, 11.1 mg CaCl$_2$) culture were grown containing 0.4% glucose, 5 mL of 200 × vitamin solution (per liter: 200 mg biotin, 200 mg choline chloride, 200 mg folic acid, 200 mg of nicotinic acid, 200 mg of pantothenate, 200 mg of pyridoxal, 200 mg of riboflavin, pH adjusted to 7), 0.5 mL of 0.1% thiamine, and a 50 μM MnCl$_2$, FeCl$_3$, or ZnCl$_2$ metal supplement.

Cultures in LB (350 mL) were grown at 37 °C until the absorbance at 600 nm was ~1.00. Cultures were then induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the temperature shifted to 27 °C. After 21 h, the cells were harvested and washed three times with 100 mL of Chelex-treated 50 mM Tris-Cl, pH 7.00, by resuspension followed by centrifugation at 11 900 × g for 20 min at 4 °C. Crude extract was prepared by resuspending 0.5 g wet cell paste in 5 mL of 0.1 M Tris-Cl, pH 8.00, containing 4 mM phenylmethylsulfonyl fluoride (PMSF) and 0.4 mg/mL lysozyme (Sigma), incubated for 3 h at 4 °C; homogenized (Ultra-Turrax T8, IKA Labortecnik) three times for 30 s, and centrifuged at 23 700g (4 °C) for 45 min. The supernatant was used directly for phosphatase assays and protein concentration determination (described below). Cultures in minimal media were grown and cell lysate prepared as described above, with the exception that induction was performed for 14 h.

**Metal Analysis.** Concentrated nitric acid (11 M) was added to an aliquot of the supernatant prepared as above to a final concentration of 1 M. The suspension was incubated overnight at 4 °C and then centrifuged at 16 000g, 4 °C, for 15 min. Each supernatant was diluted in 0.1 M Tris-Cl, pH 8.0 for metal analysis. Mn and Ni analyses were performed using atomic absorption spectroscopy (Perkin-Elmer 3100, Shelton, CT), and Fe, Zn, Cu, Ca, and Mg analyses were performed using Inductively Coupled Plasma Emission Spectroscopy (ICP-ES) in the Mayo Metals Laboratory. Metal concentrations reported represent the concentration in crude cell extract.

**Metal Stoichiometry.** The metal stoichiometry of $\lambda$PP in cell lysate was determined from the metal concentrations, determined as described above, and an estimation of the concentration of $\lambda$PP, determined by comparing the Coomassie blue-stained intensity of an aliquot of cell lysate against known quantities of purified $\lambda$PP by use of SDS-PAGE. For each gel, the intensity of bands corresponding to $\lambda$PP were analyzed using ImageQuant software (Molecular Dynamics), and a standard curve of intensity versus micrograms of purified $\lambda$PP was used to estimate the concentration of $\lambda$PP in cell lysate.

**Phosphatase Activity Assays.** Cell lysate prior to nitric acid treatment was assayed for phosphatase activity in 0.1 M Tris-Cl, pH 7.80, using 20 mM p-nitrophenylphosphate (pNPP) as substrate. Protein formation was measured spectrophotometrically at 410 nm using ΔA$_{410}$ = 14 400 M$^{-1}$ cm$^{-1}$ at pH 7.8. It was necessary to dilute cell lysate from overexpressing cells 20-fold in 0.1 M Tris-Cl, pH 7.80, prior to use in the phosphatase assays but there was no need to dilute the lysate from nonexpressing control cells. Protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) using BSA as a standard.

**EPR Spectroscopy.** Cultures (75 mL) of *E. coli* BL-21 Star (DE3) cells transfected with $\lambda$PPpT77 or control cells without this plasmid were grown with metal supplementation in LB with ampicillin (0.1 mg/mL) or LB, respectively, and induced with IPTG as described above. After 21 h, 40 mL of each culture was centrifuged at 23 700g, 4 °C, for 5 min to pellet the cells. The supernatant was discarded and each cell pellet was resuspended in 800–825 μL of 0.1 M Tris-Cl, pH 8.00 (Chelex-treated), to normalize samples to equivalent optical densities at 600 nm. An aliquot (250 μL) of each cell suspension was transferred to a quartz EPR tube and frozen by immersion in liquid nitrogen. EPR spectra were recorded using a Bruker ESP 300E spectrometer operating at X-band microwave frequency equipped with an Oxford Instruments ESR 900 continuous-flow cryostat for cryogenic temperature regulation. Signal averaging to improve signal-to-noise was performed by averaging 20 scans for each EPR sample of whole cells. Samples of mononuclear Mn$^{2+}$- and dinuclear ([Mn$^{3+}$]$_2$)-$\lambda$PP were prepared as described (43). Following desalting, the mononuclear Mn$^{2+}$-$\lambda$PP sample had an Mn/protein ratio of 0.63. The dinuclear ([Mn$^{3+}$]$_2$)-$\lambda$PP sample was prepared by addition of two equivalents of Mn$^{2+}$ to the enzyme sample.

Estimation of the concentration of mononuclear Mn$^{2+}$- and dinuclear ([Mn$^{3+}$]$_2$)-$\lambda$PP in whole cell EPR samples was performed by comparing the intensity of specific EPR signals in the spectra from intact *E. coli* cells to the corresponding signals observed in spectra of mononuclear-Mn$^{2+}$ and dinuclear-[Mn$^{3+}$]$_2$ forms of $\lambda$PP, prepared as described previously (43, 44). EPR spectra were measured at equivalent microwave power and temperature and corrected for gain and number of scans.

**RESULTS**

**Metal Content of *E. coli* Cells Following Overexpression of Bacteriophage $\lambda$ Protein Phosphatase.** $\lambda$PP was overexpressed in *E. coli* using a T7 expression system as described
Table 1: Metal Concentrations of Crude Extract from Control (pT77 and BL-21 Star) and Overexpressing (pPpT77) Cells Grown in LB Medium with a Multiple Metal Supplement

<table>
<thead>
<tr>
<th>metal</th>
<th>control cells [metal] (μM)</th>
<th>overexpressing cells [metal] (μM)</th>
<th>average fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn</td>
<td>2.6 ± 1.5</td>
<td>58.4 ± 19.3</td>
<td>22.5×</td>
</tr>
<tr>
<td>Ca</td>
<td>96.9 ± 20.2</td>
<td>114.6 ± 21.3</td>
<td>1.2×</td>
</tr>
<tr>
<td>Mg</td>
<td>211.9 ± 42.1</td>
<td>339.5 ± 98.6</td>
<td>1.6×</td>
</tr>
<tr>
<td>Cu</td>
<td>1.45 ± 0.51</td>
<td>1.57 ± 0.49</td>
<td>1.1×</td>
</tr>
<tr>
<td>Zn</td>
<td>11.0 ± 2.4</td>
<td>62.5 ± 17.7</td>
<td>5.7×</td>
</tr>
<tr>
<td>Fe</td>
<td>15.2 ± 4.1</td>
<td>64.9 ± 14.5</td>
<td>4.3×</td>
</tr>
<tr>
<td>Ni</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>1.0×</td>
</tr>
</tbody>
</table>

*a* Metal concentrations were measured using inductively coupled plasma emission spectroscopy (ICP-ES), except Mn and Ni, which were determined by atomic absorption spectroscopy. Experiments were repeated with \( n = 19-21 \) for control cells and \( n = 17-18 \) for cells overexpressing \( \lambda PP \), respectively, with the mean ± 0.5 standard deviation shown. Values marked with “<” represent samples which had metal concentrations below the detection limit.

Previously (27), cells that either did not contain this plasmid or that were transfected with the parent plasmid (pT7-7) were prepared in a similar fashion and used as the negative control. Crude extracts from control and overexpressing cells were prepared and the concentrations of Ca, Cu, Fe, Mg, Mn, Ni, and Zn were measured (Table 1). The metal concentrations of fifteen preparations of BL-21 Star (DE3) pLys transformed with pT7-7, and six preparations of this strain without pT7-7 were found to be statistically equivalent and are therefore grouped together in the column marked “Control Cells” in Table 1.

Mn showed the greatest increase upon overexpression of \( \lambda PP \) (Table 1). In six separate preparations, the Mn content of crude extract increased from 9- to 48-fold, for an average 22.5-fold increase compared to control cells (complete experimental data provided in Supporting Information). Fe and Zn concentrations also increased following overexpression of \( \lambda PP \), with average increases of 4.3- and 5.7-fold, respectively. A smaller increase was observed for Mg (1.6-fold increase), whereas Ca and Cu both showed slight decreases. Metal stoichiometries were determined by comparing metal concentrations of crude cell lysate with an estimation of the concentration of \( \lambda PP \) in crude lysate as described in the Methods. The average Mn/\( \lambda PP \) molecule metal stoichiometry was found to be 0.34 ± 0.04 (\( n = 7 \)).

When cells overexpressing \( \lambda PP \) were grown in LB media in the absence of the multimetal supplement, only modest changes in intracellular metal concentrations were observed, with Mn, Ca, Zn, and Fe increasing ≤2-fold (data not shown). The metal content of cells overexpressing \( \lambda PP \) following growth in minimal media selectively supplemented with 50 \( \mu \)M Mn\(^{2+}\), Zn\(^{2+}\), or Fe\(^{2+}\) was also assayed (Table 2). Modest increases in Mn, Zn, and Fe of 2.3-, 2.7-, and 1.6-fold were observed.

**Phosphatase Activities of E. coli Crude Extract Overexpressing \( \lambda PP \).** The question of which metal is bound to \( \lambda PP \) in vivo can be partially addressed by phosphatase assays. Phosphatase assays were performed on freshly prepared cell lysates from overexpressing and control (i.e., those transfected with pT7-7) cells when grown on LB medium with (Table 3A) and without (Table 3B) metal supplementation, and measured in the presence or absence of exogenous 1 mM Mn\(^{2+}\). Basal phosphatase activities of control cell lysates grown with and without metal supplementation are comparable. In the absence of exogenous Mn\(^{2+}\), the phosphatase activity of lysate from cells overexpressing \( \lambda PP \) is 850-fold higher than basal phosphatase activity in control cells when both are grown in the presence of a multi-metal supplement (Table 3A) indicating that metal-activated enzyme exists in lysate from the former. In contrast, the phosphatase activities in the absence of exogenous Mn\(^{2+}\) prepared from cells grown without metal supplementation show a less significant
Mn$^{2+}$ is a Native Metal for Bacteriophage λPP

DISCUSSION

With examples found throughout all five taxonomic kingdoms, the protein motif that provides scaffolding for the active site metal ions of λPP is one of the most highly distributed motifs in nature. One unresolved feature of many enzymes in this class is the identity of the intrinsic metal ion cofactor(s). A corollary to this is whether the same metal ion cofactors are used in different organisms; i.e., is the requirement for specific metal ions also conserved throughout evolution? The identity of the physiologically relevant metal ion is often unknown because most structure/functions studies rely upon recombinant proteins often purified using buffers containing EDTA or supplemented with exogenous divalent metal ions. To date, only a handful of enzymes from this
class have been purified from native sources and characterized with respect to their intrinsic metal ions. Purple acid phosphatases purified from mammalian tissue, e.g., porcine uterus and bovine spleen, contain a dinuclear iron center (20, 21), whereas plant purple acid phosphatases contain Fe–Zn or Fe–Mn centers (22–24). Collinearin purified from bovine brain is an Fe–Zn enzyme (37, 47). It is also likely that protein phosphatase 2A is an Fe–Zn metalloenzyme (48). None of the bacterial protein phosphatases have been purified containing native metals, although some, such as λPP and PrpA and PrpB from *Salmonella enterica* have been shown to be activated by a number of divalent metal ions, with Mn$^{2+}$ and Ni$^{2+}$ providing the highest specific activity (27, 42).

In this manuscript, we provide evidence that Mn$^{2+}$ is the physiological activator for λPP. We have resorted to using an overexpression system to increase the yield of protein in cell lysate such that changes in intracellular metal content upon overexpression can be measured. Overexpression of λPP resulted in a sizable (22.5-fold) increase in intracellular [Mn$^{2+}$]. Compared to the extracellular concentration of Mn$^{2+}$ of 4 μM (in cultures grown in LB media), *E. coli* cells overexpressing λPP concentrate Mn$^{2+}$ 200-fold. Furthermore, λPP in crude extracts is active in the absence of exogenous divalent metal ions, indicating the presence of metal-activated enzyme. In all preparations in which λPP is overexpressed, Cu and Ni are present at substoichiometric quantities (<0.01 equivalents), thus ruling out these metal ions as being responsible for the phosphatase activity observed in crude extracts. The other metals tested (Mg, Mn, Ca, Zn, and Fe) are present at near-stoichiometric concentrations but Mg$^{2+}$, Zn$^{2+}$, and Fe$^{3+}$ do not activate λPP (27). Thus, only Mn and Ca, which are present in near-stoichiometric quantities compared to λPP, can be responsible for the observed phosphatase activity in crude extract. Of these two, Mn$^{2+}$ is a more potent activator (~17×, see ref 27) and is the only one of these two metals which has a significant increase in intracellular concentration upon overexpression of λPP. Metal analyses of cells grown in minimal media with singular additions of exogenous Mn, Zn, or Fe show only modest increases in the cytoplasmic concentration of these metals (2.3-, 2.7, and 1.6-fold, respectively). These experiments indicate that even when provided individually, Fe and Zn do not accumulate as much as Mn does in metal-supplemented LB media overexpressing λPP.

The choice of metal ion(s) utilized by a metallolprotein in a given organism is governed by a number of factors including environmental availability, the presence of specific transport systems, thermodynamic and kinetic considerations regarding metal binding and dissociation, and whether specific metal-chaperoning systems are required for metal insertion. In *Salmonella typhimurium* and *E. coli*, the NRAMP protein MntH is likely to be involved in metal uptake by λPP overexpressing cells (49). In *E. coli*, transcription of MntH is regulated by both the Fur protein, which functions as a repressor in its Fe- or Mn-replete state (49) and the Mn-dependent repressor, MntR (50). Overexpression of λPP may transiently lower cytosolic Mn$^{2+}$ concentration and result in derepression of Fur- and MntR-regulated genes and upregulation of MntH. MntH, which has a higher affinity for Mn$^{2+}$ than for Fe$^{2+}$ or Zn$^{2+}$, with $K_{m}$ of 1, 100–200, and >100 μM, respectively (49), will likely result in an increase in cellular Mn. Curiously, when Mn$^{2+}$ alone is provided to *E. coli* cells overexpressing λPP in a defined medium, only a modest 2.3-fold increase in intracellular Mn occurs, suggesting that Mn$^{2+}$ import may require the presence of other exogenous metal ions. The involvement of both Fe and Mn in the transcriptional regulation of MntH (described above) (50) indicates that divalent metal transport may often be cooperatively regulated by several different metal ions.

It is worth noting that in addition to Mn, Fe and Zn concentrations also increase upon λPP overexpression. Zn transport is regulated by the Zn-dependent repressor Zur. In its Zn-replete state, Zur inhibits transcription of the znuABC gene locus, which encodes a Zn transporter (51). Fe transport is regulated by the Fur repressor, which in its iron-bound form inhibits genes for outer membrane proteins and also those involved in siderophore biosynthesis (52). An argument could be made that the use of a multimetal supplement could overwhelm transport of Fe$^{2+}$ and Zn$^{2+}$, which might be the real in vivo activators of λPP. However, neither Fe(II) nor Zn(II) activate λPP as noted above. More importantly, the Fe and Zn content of cells grown in minimal media supplemented individually with these metal ions showed only modest (~2-fold) increase in their respective concentrations, ruling out Mn(II) as an inhibitor of Fe or Zn uptake. The fact that Mn, Fe, and Zn import are regulated by different transcriptional repressors, and of these three, Mn uptake increases the most upon λPP overexpression, indicates that the intracellular Mn concentration becomes significantly depleted in *E. coli* under these conditions.

The majority of spectroscopic studies on metalloenzymes utilize homogeneously purified and highly concentrated enzyme samples. Although these studies provide detailed structural and mechanistic information, one can be left with questions of physiological relevance and whether the information from in vitro studies correctly represents the protein’s mechanism of action within the cell. Whole cell spectroscopy can provide significant information on the metal content, redox states, and dynamics of metalloenzyme action in the cell (53–56). However, high (or over-) expression and significant metalation of an enzyme are necessary in order to observe and discern resonances above the background signals of whole cells, making the use of this technique rare. While such high protein overexpression may not be deemed physiological, such techniques are often employed to gain useful information on an enzyme’s metallation/oxidation state in vivo. EPR spectroscopy of intact *E. coli* cells overexpressing λPP provides evidence that Mn$^{2+}$ is bound directly to λPP inside the cells, as noted by the appearance of resonances previously attributed to mononuclear Mn$^{2+}$ and dinuclear ([Mn$^{2+}$]$^{2+}$) species of λPP.

λPP is an enzyme that has been shown to be activated by a variety of divalent metals including Mn$^{2+}$ and Ni$^{2+}$ in vitro (27). EPR spectroscopy (43, 44) and X-ray crystallography (I) have verified that λPP is capable of binding two atoms of Mn$^{2+}$ in its active site. Despite these previous studies, the identity of the activating metal in vivo had not been resolved. The best activating metal for an enzyme in vitro may not be bioavailable or correctly targeted for insertion into the apo-enzyme in vivo. Through a combination of whole cell EPR spectroscopy, kinetic measurements, and metal analysis we have shown that λPP can effectively bind and become activated by Mn$^{2+}$ in vivo.


**ACKNOWLEDGMENT**

This article is dedicated to Frank Rusnak, who passed away September 7th, 2002, and his wife and young son, Rae and Leo.

**SUPPORTING INFORMATION AVAILABLE**

Metal analyses of six preparations of cells overexpressing λPP grown in LB with metal supplementation. This material is available free of charge via the Internet at http://pubs.acs.org.

**REFERENCES**