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On the mechanism of *Escherichia coli* pyridoxal kinase inhibition by pyridoxal and pyridoxal 5′-phosphate☆

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ABSTRACT

Pyridoxal 5′-phosphate (PLP), the catalytically active form of vitamin B_6, plays a crucial role in several cellular processes. In most organisms, PLP is recycled from nutrients and degraded B_6-enzymes in a salvage pathway that involves pyridoxal kinase (PLK), pyridoxine phosphate oxidase and phosphatase activities. Regulation of the salvage pathway is poorly understood. *Escherichia coli* possesses two distinct pyridoxal kinases, PLK1, which is the focus of the present work, and PLK2. From previous studies dating back to thirty years ago, pyridoxal (PL) was shown to inhibit *E. coli* PLK1 forming a covalent link with the enzyme. This inhibition was proposed to play a regulative role in vitamin B_6 metabolism, although its details had never been clarified. Recently, we have shown that also PLP produced during PLK1 catalytic cycle acts as an inhibitor, forming a Schiff base with Lys229, without being released in the solvent. The question arises as to which is the actual inhibition mechanism by PL and PLP. In the present work, we demonstrated that also PL binds to Lys229 as a Schiff base. However, the isolated covalent PLK1–PL complex is not inactive but, in the presence of ATP, is able to catalyse the single turnover production of PLP, which binds tightly to the enzyme and is ultimately responsible for its inactivation. The inactivation mechanism mediated by Lys229 may play a physiological role in controlling cellular levels of PLP. This article is part of a Special Issue entitled: Cofactor-dependent proteins: evolution, chemical diversity and bio-applications.

1. Introduction

Pyridoxal 5′-phosphate (PLP), the catalytically active form of vitamin B_6, is the cofactor of a great number of enzymes involved in central metabolic pathways [1–3]. In *Escherichia coli*, PLP is synthesised following the so-called deoxyxylulose 5-phosphate (DXP)-dependent biosynthetic pathway [4]. The final product of this route is pyridoxine 5′-phosphate (PNP), which is then oxidized to PLP by PNP oxidase [5,6]. In addition, *Escherichia coli*, as many other prokaryotic and eukaryotic organisms, utilizes a salvage pathway in which all B_6 vitamers coming from growing medium and protein turnover can be converted into PLP by the concerted action of PNP oxidase, phosphatases and kinases [7–9]. *E. coli* possesses two different kinases that are able to phosphorylate pyridoxal (PL) [10]. *E. coli* PLK1 (*e*PLK1), the object of this study, is encoded by the *pdxK* gene and is also able to phosphorylate pyridoxine (PN), pyridoxamine (PM) and hydroxymethylpyrimidine [11], although *E. coli* has a specific kinase for this latter substrate [12]. *E. coli* PLK2 (*e*PLK2), encoded by the *pdxY* gene, is specific for PL [13].

About thirty years ago, it had been proposed that pyridoxal is capable to inhibit *E. coli* PLK1 binding to it as Schiff base to a lysine residue [14,15]. However, this lysine residue had not been identified. PL inhibition took place in the absence of ATP and therefore was not related to the enzyme catalytic turnover. We have recently observed that the enzyme also undergoes inhibition while catalysing the ATP-dependent phosphorylation of pyridoxal, forming a tight complex with PLP through an aldimine linkage with residue Lys229 [16]. In this case, PLP that binds to *e*PLK1 and is responsible for its inactivation must be produced at the active site of the enzyme as a consequence of

Abbreviations: PLP, pyridoxal 5′-phosphate; PNP, pyridoxine 5′-phosphate; PN, pyridoxine; PL, pyridoxal; *e*PLK1, *E. coli* pyridoxal kinase encoded by *pdxK* gene; *e*PLK2, *E. coli* pyridoxal kinase encoded by *pdxY* gene; *sa*PLK, *Staphylococcus aureus* pyridoxal kinase

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its catalytic turnover. Although PLP is tightly bound to the inactivated enzyme and cannot easily be removed, it can be transferred to a PLP-dependent apoenzyme with consequent reactivation of *e*PLK1 [16].

Given the observations on *e*PLK1 inhibition by either substrate or product, the question arises as to whether PL and PLP actually inhibit the enzyme following two different mechanisms. Whether the regulation of enzyme activity, and therefore the availability of PLP through the salvage pathway, is based on these observed inhibition processes is still a main point to be elucidated. In this work, we present a series of experiments carried out to clarify the mechanism of inhibition of *e*PLK1 by PL and PLP and also the role of Lys229 in enzyme inhibition and catalysis.

2. Materials and methods

2.1. Materials

Ingredients for bacterial growth were from Fluka. Ni-NTA Agarose for purification of 6xHis-tagged proteins was from Qiagen Inc. (Valencia, CA, USA). All other chemicals were from Sigma-Aldrich (St. Louis, MO, USA) and Carlo Erba (Milano, Italy). Wild type and K229Q *e*PLK1 were purified as previously described [16]. *E. coli* bacterial strains BW25113 (wild-type), JW1628 (Δ*pdxY*) and JW2411 (Δ*pdxK*) used for cell extracts assays were obtained from the *E. coli* Genetic Stock center (Yale University, New Haven, CT, USA).

2.2. Activity assays

Activity assays and all other experiments were carried out in 20 mM potassium HEPES, pH 7.5, containing 0.5 mM $MgCl_2$. The catalytic activity of *e*PLK1 was determined spectrophotometrically at 37 °C by observing the initial increase in absorbance at 388 nm during the conversion of PL to PLP [13]. The concentration of PLP produced by the enzyme in potassium HEPES buffer at pH 7.5 was measured using an extinction coefficient at 388 nm of 5020 M^{-1} cm^{-1}. This extinction coefficient was calculated as the difference between the extinction coefficients of PLP and PL, determined independently from solutions whose concentrations were previously measured in NaOH [17]. Kinetic measurements in the activity assays were performed on a Hewlett-Packard 8453 diode-array spectrophotometer. Inhibition kinetics were analysed using Eq. (1).

2.3. Isolation and stoichiometry of the ePLK1–PL complex

*e*PLK1 (150 μM) was mixed with 0.5 mM PL and incubated at 37 °C for one hour. The sample was then loaded onto a Sephadex G-50 column (1 cm Ø × 25 cm) equilibrated and then eluted with potassium HEPES buffer collecting 1 mL fractions. The first 20 fractions were analysed by measuring their absorption spectra between 250 and 500 nm to detect the presence of protein and pyridoxal. The stoichiometry of protein and PL in the *e*PLK1–PL complex was determined by adding NaOH to a final concentration of 0.2 M to denature the protein and release bound PL. The absorbance at 388 nm was used to determine the concentration of the cofactor, that exhibits a molar extinction coefficient of 1700 M^{-1} cm^{-1} [17]. The concentration of protein in the complex was also determined in 0.2 M NaOH, using an extinction coefficient at 292 nm (a wavelength at which PLP has negligible absorbance) of 35,416 M^{-1} cm^{-1}, which was determined from the absorption spectrum of a protein sample whose concentration was previously determined in buffer (extinction coefficient at 278 nm of 27,910 M^{-1} cm^{-1}) [13].

2.4. Analysis of PL and PLP binding equilibria

Analyses took advantage of the protein intrinsic fluorescence quenching observed upon binding event. Dissociation constants of binding equilibria were then calculated from saturation curves obtained measuring the protein fluorescence emission intensity as a function of increasing ligand concentration. Either PL or PLP (from 1 to 100 μM) was added to enzyme samples (0.5 μM subunit concentration) at 37 °C in 20 mM potassium HEPES at pH 7.5, containing 0.5 mM $MgCl_2$. Preliminary experiments demonstrated that the binding equilibrium was established within 10 min. Fluorescence emission measurements were carried out at 37 °C with a FluoroMax-3 Jobin Yvon Horiba spectrofluorimeter using a 1-cm path length quartz cuvette. Fluorescence emission spectra were recorded from 300 nm to 450 nm with the excitation wavelength set at 280 nm. Excitation and emission slits were set at 5 nm. Fluorescence emission spectra of samples containing the same concentration of ligand (PL or PLP) but without *e*PLK were also recorded and subtracted from the respective spectra with *e*PLK at each titration point. Data were analysed according to Eq. (3).

2.5. Data analysis

Inhibition kinetics were analysed using Eq. (1) in which [*PLP*] is the observed *PLP* concentration, $[PLP]_{max}$ is PLP concentration produced after complete enzyme inactivation and k_i is the observed inhibition rate constant.

$$[PLP] = [PLP]_{max} \times \left(1 - e^{-k_i \times t}\right) \quad (1)$$

The observed inhibition rate constant shown in Fig. 2 (lower panel) were analysed according to Eq. (2), in which k_{obs} is the observed inhibition constant, k_{max} is the maximum inhibition constant, [*PL*] is pyridoxal concentration and K_d is an apparent dissociation constant.

$$k_{obs} = k_{max} \times \frac{[PL]}{[PL] + K_d} \quad (2)$$

Fluorescence measurements obtained from binding equilibria experiments were transformed in fractional variation with respect to the extrapolated maximum change at infinite ligand concentration and then analysed according to Eq. (3) in which *f* is the observed fluorescence fractional variation, [*L*] is the ligand and K_d the dissociation constant.

$$f = \frac{[L]}{L + K_d} \quad (3)$$

All data were analysed using the software Prism (GraphPad Software Inc., San Diego, CA, USA).

2.6. Activity measurement on cell extracts

Starter cultures of BW25113 (wild-type), JW1628 (Δ*pdxY*) and JW2411 (Δ*pdxK*) (*E. coli* Genetic Stock center) were grown overnight at 37 °C in LB medium. Overnight cultures (2 mL) were used to inoculate 200 mL of fresh LB containing kanamycin (for the knock out mutant strains). The cultures were grown with shaking at 37 °C until OD_{600} reached 1.2 and were then harvested by centrifugation at 9000 rpm for 15 min at 4 °C. Cells were suspended in 20 mL of 20 mM potassium HEPES buffer at pH 7.5, containing 0.5 $MgCl_2$ and centrifuged again. This washing step was repeated twice to remove any residual of growth medium, ending with cells suspended in 10 mL of buffer. Cells were disrupted by sonication and centrifuged at 12.000 rpm for 15 min at 4 °C. Supernatants were assayed for PL kinase activities. PL kinase activity was measured by the fluorimetric assay as described in [10,18]. Reaction mixtures (2 mL) contained 1 mM MgATP, 0.5 mM $MgCl_2$, 0.2 mM PL and 200 μL of crude extract in 20 mM potassium HEPES buffer. Reactions were started by addition of crude extract and incubated at 37 °C. At time intervals, the sample was quickly transferred into a thermostated cuvette at 37 °C under continuous stirring.

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