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Review

Vitamin B_6 salvage enzymes: Mechanism, structure and regulation ☆

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ABSTRACT

Vitamin B_6 is a generic term referring to pyridoxine, pyridoxamine, pyridoxal and their related phosphorylated forms. Pyridoxal 5′-phosphate is the catalytically active form of vitamin B_6, and acts as cofactor in more than 140 different enzyme reactions. In animals, pyridoxal 5′-phosphate is recycled from food and from degraded B_6-enzymes in a "salvage pathway", which essentially involves two ubiquitous enzymes: an ATP-dependent pyridoxal kinase and an FMN-dependent pyridoxine 5′-phosphate oxidase. Once it is made, pyridoxal 5′-phosphate is targeted to the dozens of different apo-B_6 enzymes that are being synthesized in the cell. The mechanism and regulation of the salvage pathway and the mechanism of addition of pyridoxal 5′-phosphate to the apo-B_6-enzymes are poorly understood and represent a very challenging research field. Pyridoxal kinase and pyridoxine 5′-phosphate oxidase play kinetic roles in regulating the level of pyridoxal 5′-phosphate formation. Deficiency of pyridoxal 5′-phosphate due to inborn defects of these enzymes seems to be involved in several neurological pathologies. In addition, inhibition of pyridoxal kinase activity by several pharmaceutical and natural compounds is known to lead to pyridoxal 5′-phosphate deficiency. Understanding the exact role of vitamin B_6 in these pathologies requires a better knowledge on the metabolism and homeostasis of the vitamin. This article summarizes the current knowledge on structural, kinetic and regulation features of the two enzymes involved in the PLP salvage pathway. We also discuss the proposal that newly formed PLP may be transferred from either enzyme to apo-B_6-enzymes by direct channeling, an efficient, exclusive, and protected means of delivery of the highly reactive PLP. This new perspective may lead to novel and interesting findings, as well as serve as a model system for the study of macromolecular channeling. This article is part of a Special Issue entitled: Pyridoxal Phosphate Enzymology.

1. Introduction

Vitamin B_6 is a generic term which actually refers to the ensemble of six interconvertible pyridine compounds (vitamers): pyridoxine (PN, commonly known as vitamin B_6), pyridoxamine (PM), pyridoxal (PL) and their 5′-phosphorylated forms (PNP, PMP and PLP, respectively), which differ in the identity of the chemical group present at the 4′ position (Fig. 1). PLP is the biologically active and best known vitamer form, since it is used as enzyme cofactor in an enormous variety of biochemical transformations. In few enzymes, PMP also plays a catalytic role. In recent years, an additional function of B_6 vitamers as oxygen reactive species (ROS) scavengers and factors able to increase resistance to biotic and abiotic stress has been demonstrated in plants [1,2]. PLP and PN may also function as regulators of membrane ion transporters [3–5], and have been found to bind to steroid receptors [6] and to modulate transcription factors [7].

1.1. Biosynthesis and recycling of vitamin B_6

All living beings rely on vitamin B_6 for their existence, however, only microorganisms and plants are able to synthesize it *de novo*. All other organisms acquire vitamin B_6 from nutrients and interconvert its different forms in order to match their needs. Two independent *de novo* biosynthetic routes are known (Fig. 2) [8]. The first to be discovered was extensively studied in *Escherichia coli* and for a long time assumed to be ubiquitous. Nowadays, we know it is restricted to some eubacteria. This pathway, also called DXP-dependent pathway, is articulated in two branches which, starting from 4-phosphohydroxy-L-threonine (derived from erythrose 4-phosphate) at one end and from pyruvate and gliceraldehyde 3-phosphate at the other end, join in a ring closure reaction catalyzed by PNP synthase (coded by the

Abbreviations: PN, pyridoxine; PM, pyridoxamine; PL, pyridoxal; PNP, pyridoxine 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PLP, pyridoxal 5′-phosphate; PLK, pyridoxal kinase coded by *PdxK* gene; PLK2, pyridoxal kinase coded by *PdxY* gene; PNPOx, pyridoxine (pyridoxamine) 5′-phosphate oxidase; NEE, neonatal epileptic encephalopathy; SHMT, serine hydroxymethyltransferase; AAT, aspartate aminotransferase

☆ This article is part of a Special Issue entitled: Pyridoxal Phosphate Enzymology.

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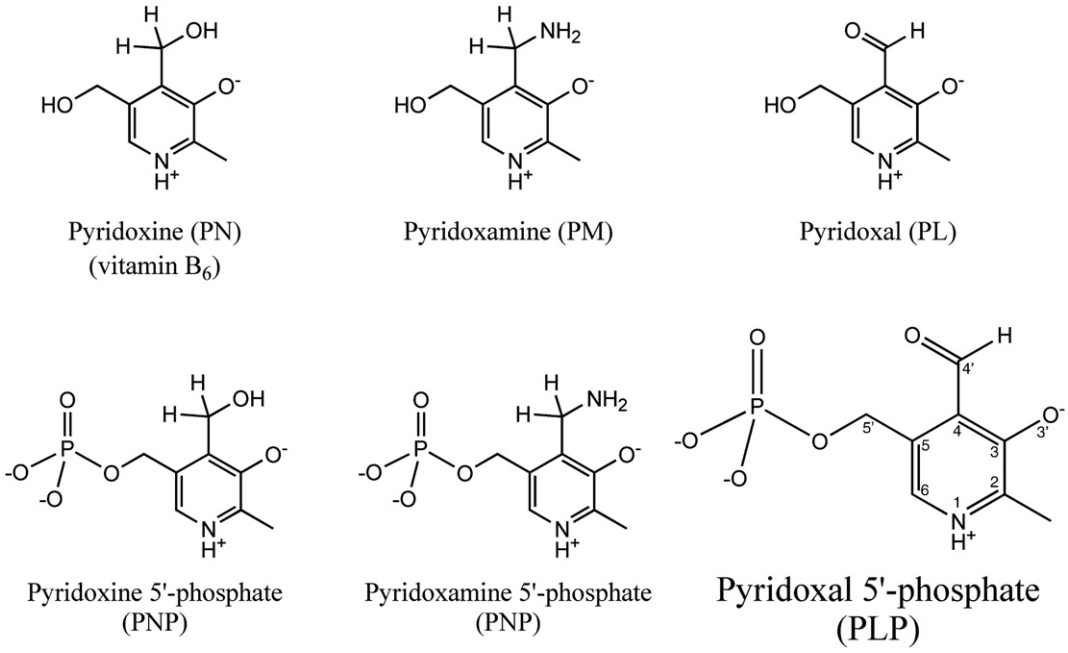

Fig. 1. Structures of the six B_6 vitamers. Carbon atom numbering is shown on the PLP structure.

PdxJ gene), which forms PNP, the first B_6 vitamer to be synthesized [9]. In the second route, the so-called DXP-independent pathway, PLP is directly formed from glutamine, either ribose or ribulose 5-phosphate and either glyceraldehydes 3-phosphate or dihydroxyacetone phosphate by the action of the PLP synthase complex (coded by the Pdx1 and Pdx2 genes) [10,11]. After the second route was serendipitously discovered in fungi, it became clear that it is much more widely distributed than the first one, being found in Archaea, most eubacteria and plants [2,12].

Humans, like all other mammals, obtain PLP from B_6 vitamers acquired from diet and recycled in a "salvage pathway" involving phosphatases, an ATP-dependent pyridoxal kinase (PLK) and a flavin mononucleotide (FMN)-dependent pyridoxine (pyridoxamine) 5′-phosphate) 5′-phosphate oxidase (PNPOx) (Fig. 2) [13]. PLK phosphorylates the 5′ alcohol groups of PN, PL and PM to form PNP, PLP and PMP respectively. PNP and PMP are further oxidized to PLP by PNPOx. PLP is largely present as such in meat, associated with glycogen phosphorylase in muscles, together with smaller amounts of PMP. PN, PNP and pyridoxine glucosides are the vitamers in plants. In mammals, ingested phosphorylated B_6 vitamers are first hydrolyzed to PL, PM and PN by intestinal phosphatase, while pyridoxine glucosides are hydrolyzed by a glucosidase prior to absorption. The absorbed vitamers are rapidly cleared, mainly by uptake into the liver, where they are phosphorylated by PLK, with PNP and PMP further oxidized to PLP by PNPOx. PLP re-enters the circulation bound to a lysine residue of albumin [14]. Delivery of active cofactor to the tissues, however, requires hydrolysis of circulating PLP to PL by the ecto-enzyme tissue nonspecific alkaline phosphatases [15]. A PLP specific phosphatase is also present, with essential role in cellular metabolism, especially in the brain where its level is substantially higher [16]. Once entered the cells, PL is re-phosphorylated by PLK and is somehow targeted to dozens of newly synthesized apo-B_6 enzymes.

1.2. Homeostasis of vitamin B_6 and human health

More than 140 different enzyme activities based on PLP are classified by the Enzyme Commission. They are distributed over five out of the six enzyme classes and represent 4% of all known catalytic activities [17]. PLP-dependent enzymes are not only involved in the synthesis, interconversion and degradation of amino acids but also play key roles in the metabolism of neurotransmitters, one-carbon units, biogenic amines, tetrapyrrolic compounds, amino sugars, modulation of steroid receptor-mediated gene expression and regulation of immune function. Of particular interest is the role of B_6 enzymes in brain metabolism, since the synthesis of several neurotransmitters, such as γ-aminobutyric acid (GABA), dopamine, epinephrine, norepinephrine, serotonin, serine, and histamine involves B_6 enzymes. Proper functioning of PLP-dependent enzymes and thus optimal health are dependent upon adequate levels of PLP in the cell.

PLP deficiency has been implicated in several neurological and non-neurological disorders. Dietary PLP insufficiency is quite rare since most dietary sources contain vitamin B_6. Major reasons for PLP deficiency can be attributed to malfunctioning of PLK and PNPOx, which may be caused either by inherited pathogenic mutations [15,18–23] or by drug induced inhibition [15,24–31]. The latter may produce symptoms such as unconsciousness, seizures, sleeplessness, headache, restlessness, agitation, tremors, and hallucination, while the former is implicated in severe pathologies, including neonatal epileptic encephalopathy, seizures, autism, Down syndrome, schizophrenia, autoimmune polyglandular disease, Parkinson's, Alzheimer's, epilepsy, attention deficit hyperactivity disorders and learning disability.

Very high levels of vitamin B_6 may have toxic effects [32–39]. PLP contains a very reactive aldehyde group at the 4′ position, that easily forms aldimines with primary and secondary amines and for this reason is often used as a protein labeling agent. The current recommended dietary allowance of vitamin B_6 is 2 mg/day in the United States. Toxicity is observed usually when the concentration exceeds 200 mg/day [40]. The levels of B_6 could also be raised as a result of an environmental insult or genetic defects. Toxicity of vitamin B_6 is known to cause sensory as well as motor neuropathies leading to numbness in hands and feet, that are usually reversible when supplementation is stopped [41].

The pool of free PLP *in vivo* is maintained at a very low level in the body, presumably to prevent toxic buildup. Regulation of PLP synthesis by PLK and PNPOx is a proposed homeostasis mechanism. Zhao and Winkler observed inhibition of *E. coli* PNPOx activities by product PLP, with a K_i of 8 μM [42]. In another study by our group, significant MgATP substrate inhibition of *E. coli* PLK was observed in the presence of PNP or PLP [43]. However, the most well established

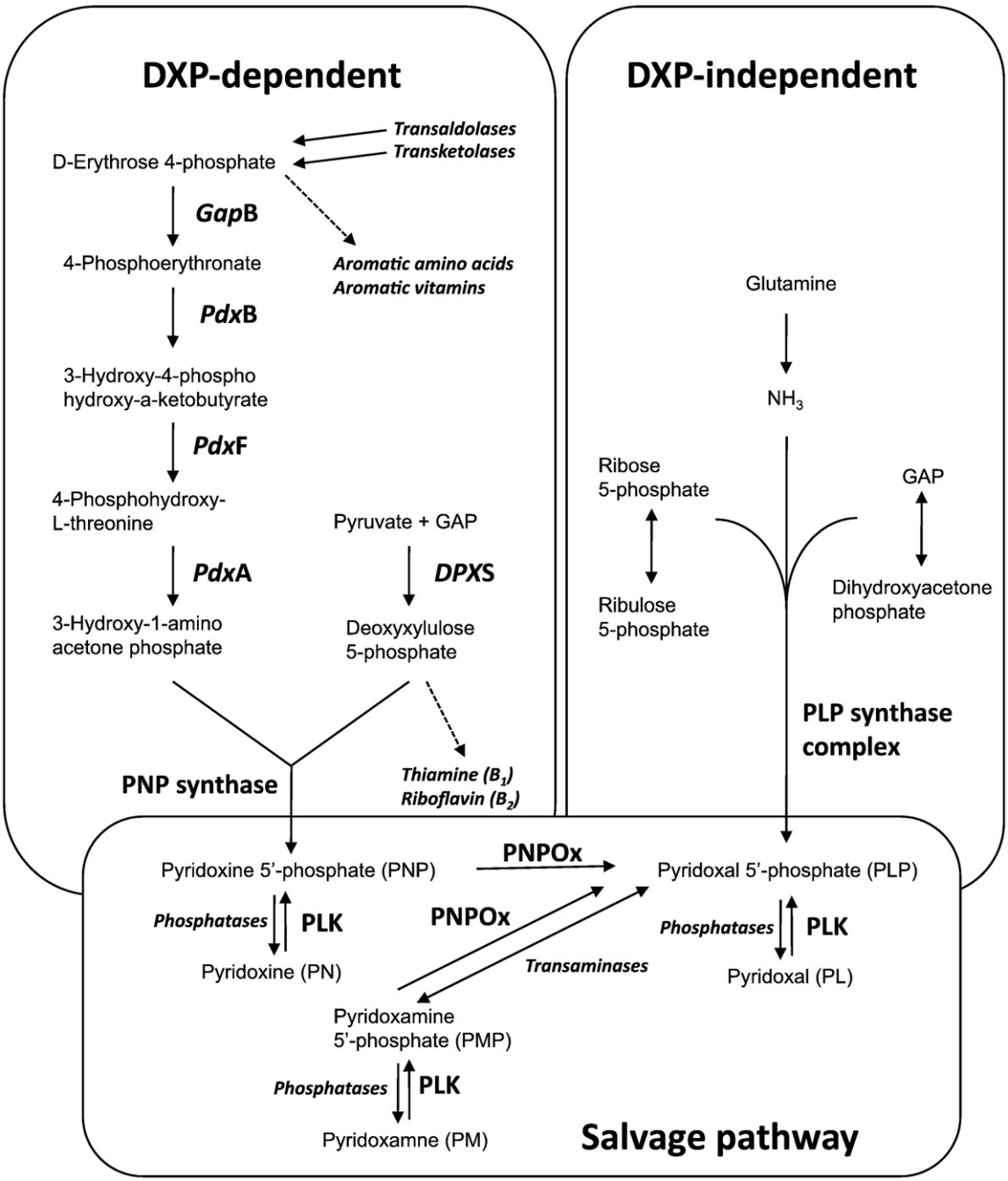

Fig. 2. Vitamin B_6 biosynthetic pathways: *De novo* DXP-dependent pathway (present in some eubacteria): *GapB*, D-erythrose 4-phosphate dehydrogenase; *PdxB*, erythronate-4-phosphate dehydrogenase; *PdxF/SerC*, phosphoserine aminotransferase; *PdxA*, 4-hydroxythreonine-4-phosphate dehydrogenase; *DPXS*, 1-deoxy-D-xylulose-5-phosphate synthase; PNP synthase, from *PdxJ* gene. *De novo* DXP-independent pathway (present in other eubacteria, fungi, plants and Archaea): PLP synthase complex: synthase domain from *Pdx1* gene; glutaminase domain from *Pdx2* gene. Salvage pathway (present in all organisms, including mammals): PLK, pyridoxal kinase from *PdxK* gene and pyridoxal kinase 2 from *PdxY* gene; PNPOx, pyridoxine 5′-phosphate oxidase from *PdxH* gene.

mechanism for maintaining low levels of free PLP is its dephosphorylation by phosphatases. Catalytic conversion of pyridoxal to 4-pyridoxic acid by aldehyde oxidase and NAD-dependent dehydrogenases is another mechanism of regulation of free PLP concentration [44]. In eukaryotic cells, the concentration of free PLP is maintained as low as 1 μM, nevertheless enough PLP is provided to B_6 enzymes. This raises the intriguing question of how the cell supplies sufficient PLP for dozens of newly synthesized apo-B_6 enzymes.

The present review deals with the two enzymes involved in the vitamin B_6 salvage pathway, PLK and PNPOx (Fig. 2). In the next two paragraphs, a description of the structural and functional characterization of these two enzymes, in relation with the mechanism and regulation of the vitamin B_6 pathway, and in relation with their involvement in several pathologies will be presented. The last paragraph, deals with the possible mechanisms by which the PLP made by these enzymes in the salvage pathway is targeted to apo-B_6 enzymes.

2. Pyridoxal kinase (PLK)

2.1. Characterization and catalytic function of PLK

Pyridoxal kinase (PLK) catalyzes the addition of phosphate from ATP to the 5′ alcohol group of PN, PM and PL to form PNP, PMP and

PLP, respectively (Fig. 3). The enzyme has been purified and characterized from various sources, including mammals, plants, insect, protozoa, and prokaryotes [45–52]. In humans, PLK is coded by a *pdxK* gene located on chromosome 21q22.3. In a study by the Winkler group with *E. coli* mutant cells that were blocked in the *de novo* biosynthetic pathway and with an inactivated *pdxK* gene, these were still able to grow on PL but not PN; this led to the discovery of a homolog PLK gene, *pdxY* [53]. While PLK from the *pdxK* gene is active toward all three unphosphorylated B_6 vitamers, the enzyme from the *pdxY* gene (PLK2) is known to phosphorylate only PL to PLP, and at a significantly low activity (less than 1% of PLK activity) [48,54]. Nevertheless, the ability to metabolize and supply PLP to the cell when the *de novo* biosynthetic pathway is blocked and in the absence of PLK has led to the proposition that PLK2 is involved in the vitamin B_6 salvage pathway; this is also supported by structural and functional studies on purified PLK2 from *E. coli* [55]. Currently, only the *pdxK* gene has been reported for eukaryotes, while most prokaryotes contain both genes. The protein data bank lists amino acid sequences for dozens of PLKs from the *pdxK* gene of both prokaryote and eukaryote origin. Sequence identity ranges from 24% to 90% when compared to the human enzyme. Several closely related PLK2 enzymes in prokaryotes are also reported. For a detailed sequence analysis of these two classes of enzymes, the reader is referred to several publications referenced here [43,55–57].

The mechanism of phosphorylation has been elucidated for both the sheep and *E. coli* PLK enzymes, and follows a random sequential substrate addition. PLK has a conserved Asp residue at the active site, with the side chain making a hydrogen-bond interaction with the C5′-hydroxyl group of the B_6 vitamer. This residue is also conserved in the ribokinase superfamily, to which PLK belongs, and was proposed to act as a catalytic base [43,46,58,59]. A recent study with D235A/N variants of human PLK is consistent with this proposition [60].

Metals, both monovalent and divalent cations, are known to be absolute requirements for the function of many kinases, providing driving forces for ATP binding and catalysis. In *E. coli* PLK, the metal ion tandem Mg^{2+} and K^+ is required for enzyme activity; the K_m values for PL and ATP are 50 μM and 450 μM, respectively, while k_{cat} is 250 min^{-1}. The corresponding kinetic parameters using PN are $K_{m(PN)} = 25$ μM, $K_{m(ATP)} = 650$ μM and $k_{cat} = 20$ min^{-1}; and using PM are $K_{m(PM)} = 30$ μM, $K_{m(ATP)} = 600$ μM and $k_{cat} = 40$ min^{-1}. Unlike PL, that occurs in significant amount in *E. coli* cells (either as a protein-bound or a free form), the levels of PN and PM are found to be negligible. This suggests that PL is the major substrate for *E. coli* PLK in the salvage pathway [48]. Zn^{2+} and K^+ had previously been proposed to be the physiological metals needed for mammalian PLK activity. However, a more recent study with the human enzyme showed that under non-physiological substrate concentrations and/or at pH 6, where these previous assays were performed, Zn^{2+} does stimulate the activity [61], but under physiological conditions at pH 7.3, Mg^{2+} is the required divalent metal ion and Zn^{2+} inhibits the reaction [48]. In a recent study with the human enzyme, Na^+ was found to elicit more than a two-fold increased enzymatic activity than K^+; however, the affinity for the ATP and PL substrates is increased many fold in the presence of K^+ as compared to Na^+. The activities of the larger cations, Rb^+, Cs^+, as well as the smallest Li^+ cation were found to be significantly lower compared to Na^+ or K^+. It has been proposed that differences in monovalent cation coordination play a role in enzyme activity and that under physiological conditions in cells, where K^+ is several-fold higher than Na^+, the enzyme is in its K^+ form [58].

2.2. Crystal structures of PLK

The crystal structures of *E. coli*, *B. subtilis*, sheep, and human PLKs and of *E. coli* PLK2, with or without substrates or products, have been elucidated [46,62–64]. The proteins are homodimers with an active site composed of residues exclusively from each respective subunit. The active site opens as a shallow groove where ATP binds, and then stretches deeper into the protein where the B_6 vitamer binds opposite but facing the γ-phosphate of the ATP (Fig. 4A). The ATP adenine moiety makes a series of van der Waals and hydrogen-bond interactions with the protein residues. The three ATP phosphate groups are also involved in extensive hydrogen-bond interactions with the protein, including a P-loop consisting of an anion hole formed by the highly conserved sequence motif GTGA (residues 232–235, human enzyme notation) and the N-terminus of a helix formed by residues 234–248. The ATP β- and γ-phosphates are further stabilized by bound Mg^{2+} and K^+ (or Na^+) ions. The interactions between the amino acid residues and metals help neutralize the negative phosphate charges and stabilize the transition state during the phosphate group transfer from ATP to the substrate. Unlike ATP, the B_6 substrate binding site is almost completely buried. The active site geometry and residues are highly conserved in all PL kinases, including PLK2. In most PLK structures, the B_6 vitamer binding site is located about 6 Å from ATP, prompting the suggestion that the protein must undergo a conformational change to a transition state that places the phosphate and the substrate closer together, to allow for transfer of the γ-phosphate from ATP to the substrate. Consistently, a ternary PLK•ADP•PLP complex structure shows such a conformational change, and it is believed to represent the catalytic conformer [43,62].

The crystal structures of Mg•ATP•PLK and Zn•ATP•PLK in complex with Na^+ and K^+ provide structural basis as to why the presence of K^+ significantly increases the affinity for ATP, even though the human enzyme is more active in the presence of Na^+. In the unliganded human PLK structure, there is a bound Na^+, which is coordinated by four or five solvent molecules. The solvent molecules make close and intricate hydrogen bond interactions with the protein residues that help stabilizing the active site conformation. Binding of ATP displaces the Na^+ to another position, closer to the protein, where it makes direct and stronger interaction with the protein, in addition to the ATP. Although the structure of the K^+-enzyme complex is unknown, it is believed that the larger K^+ (1.33 Å) would prefer more coordinating solvent molecules with relatively weaker interactions [65,66] compared to the

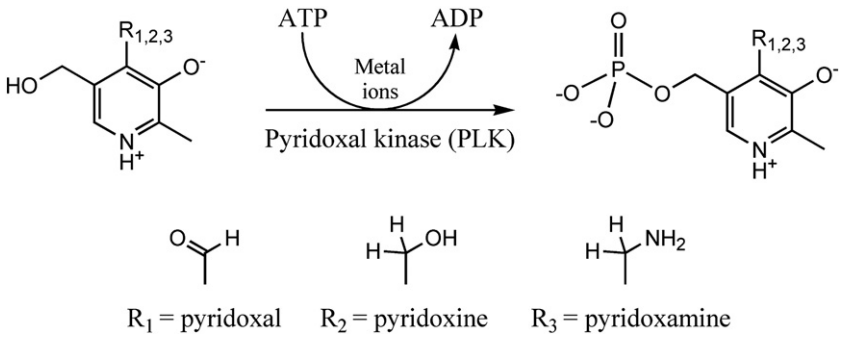

Fig. 3. Reaction catalyzed by pyridoxal kinase (PLK).

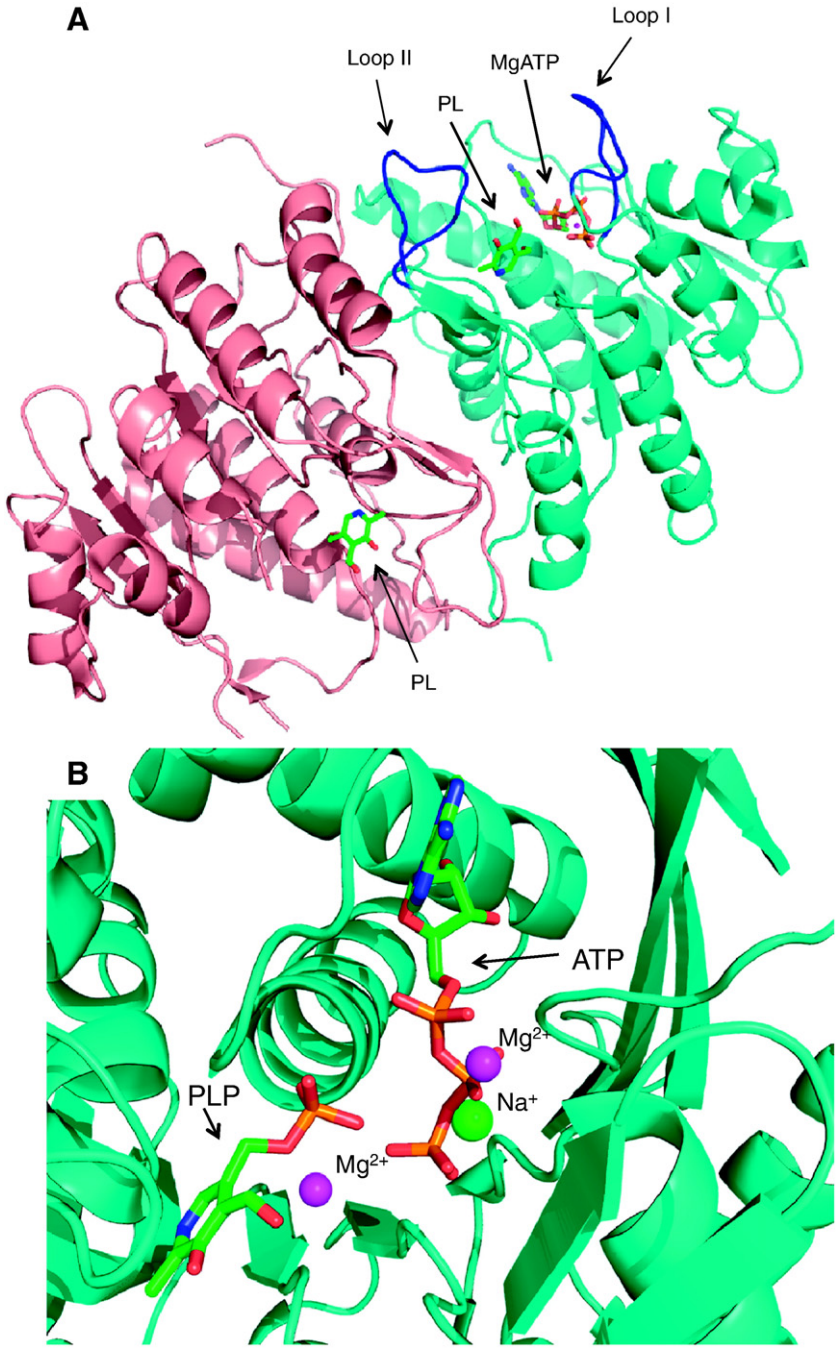

Fig. 4. Crystal structure of PLK. (A) Ribbon diagram of the *E. coli* dimeric structure of the MgATP-bound complex (PDB ID: 2DDO) superimposed with PL from the PL-bound complex (PDB ID: 2DDW). Monomer A and monomer B are in cyan and salmon, respectively. In monomer A: ATP and PL are shown as sticks with atom based colors, while Mg^{2+} is shown as a magenta sphere; loop I and loop II are shown in blue. In monomer B, PL is shown to identify the symmetry related active site, while ATP is not shown due to its disordered state. (B) Close-up view of the active site of human PLK (PDB ID: 3KEU) in a non-productive complex with MgATP, PLP and Na^+ (green sphere). Figures were generated using MacPyMOL.

smaller Na^+ (0.9 Å). The ability to strip off water molecules from the hydration sphere should therefore be easier with K^+ than with Na^+, and could explain why the enzyme shows greater affinity for ATP in the presence of K^+. The increased activity of the human enzyme in the presence of Na^+ is suggested to be due to the fact that replacement of K^+ with Na^+ changes the geometry of the active site to a more optimal orientation of catalytic residues [58].

2.3. Classification of PLK

PLKs are members of the ribokinase superfamily, showing the same typical central core tertiary structures of β-sheets surrounded by α-helices, as well as conserved ATP and substrate binding site geometries. A characteristic feature in the ribokinase superfamily structures is a lid that covers the active site. This structural feature has been suggested to be significant in substrate binding and catalysis, and has been used to propose an evolutionary pathway for the enzymes in the superfamily [57]. In all PLK structures, the lid has become a loop or a β strand-loop-β strand structure, commonly referred to as a flap (loop I in Fig. 4A). This new feature is designed to accommodate many substrates, which include PL, PM, PN, as well as other vitamin B_6 analogues. Structural and sequence comparisons of PLKs show the flap to consist of eight or nine residues in prokaryotes, differing from the 12 residues in advanced species, suggesting that the

length of the loop and possibly its conformation might serve as an indicator of evolutionary change within the PLK enzymes. A key to the random sequential kinetics exhibited by PLK is the ability to prevent unproductive hydrolysis of ATP in the absence of a bound B_6 substrate, and the flap, as well as other conserved active site residues is believed to play this important role by providing hydrogen-bond interactions to the ATP β- and γ-phosphates [46,62]. Interestingly, the flap in PLK2 rotates into the PL binding site to assume a more closed conformation, where it makes close contacts and stabilizes the bound PL. In one subunit, a conserved Cys residue on the flap of PLK2 binds covalently to the C4′ of PL as a possible thiohemiacetal, while the other subunit has both PL and PLP bound tightly in non-covalent fashion [55]. In PLKs, the flap is in a more open position and the cysteine is replaced by a hydrophobic residue. Detailed analyses of the function and evolutionary trend of the flap/lid in the ribokinase superfamily enzymes have appeared in several publications [43,46,55–57,59,67–70].

Also, central to the function and classification of the PLKs is another active site loop structure and its fingerprint Thr-Gly dipeptide (loop II in Fig. 4A). While this dipeptide is Thr47 and Gly48 in PLKs from humans, sheep and in most advanced species, there are several variations in prokaryotic PLK, with Pro-His occurring most often. In prokaryotic PLK2, the residue pair is uniquely Thr-Gln across all species. The dipeptide motif has been suggested to have a substrate binding role in sequestering the substrate for catalysis as well as determining substrate specificity [55]. While residue 47 is buried, residue 48 is located close to the mouth of the active site, and as the side-chain of the latter residue gets longer it covers the active site from the bulk solvent. Thus, the replacement of His or Gln with Gly in the human and sheep enzymes leaves the C4′ position of the substrate uncovered to the bulk solvent [43,46,55,62]. Consistently, it was shown that PLKs from rat, humans and yeast have a high tolerance to a wide variation of substitutions at the C4′ position of PL, in contrast to bacterial kinases from *Lactobacillus casei* and *Streptococcus faecalis*, which display high substrate specificity [71].

2.4. Regulatory mechanism of PLK

As already stated, PLP is a very reactive aldehyde, whom high levels in cells are known to cause motor and sensory neuropathies. On the other hand, deficiency of PLP in the cell is suspected to cause several pathologies. It is therefore clear that PLP production in the cell by PLK and PNPOx should be tightly regulated to meet the requirements for activating newly synthesized apo-B_6 enzymes. Severe inhibition of *E. coli* PLK by PLP or PNP products in the presence of substrate MgATP has been reported [43] and unpublished studies by our research group with the human enzyme are consistent with these findings. Studies using the *E. coli* enzyme show that at a fixed concentration of the substrate PL (200 μM) and product PNP or PLP (265 μM), inhibition of PLK begins to occur at 50 μM MgATP and at 100 μM, the inhibition is almost 50%. The most reasonable explanation for this inhibition is that MgATP can form a ternary complex with PLP (or PNP and perhaps PMP) at the active site. This putative non-productive PLK•PLP•ATP complex would result in the inhibition of catalytic activity. Analysis of PLK structures is consistent with the possibility that this ternary complex could form since, as noted above, the 5′-OH of PL is located ~6 Å from the γ-phosphate of ATP. In this 6 Å space there is room for the coexistence of the C5′ phosphate ester of PLP (or PNP or PMP) and the γ-phosphate of ATP. Convincing and direct evidence about formation of such an abortive ternary complex comes from a structural study of a human PLK D235A mutant, which shows ATP and PLP trapped at the active site (Fig. 4B). We have also recently determined in an unpublished study the structures of human PLK in complex with MgATP and PLP and in complex with MgATP and 4′-methylpyridoxine 5′-phosphate (phosphorylated ginkgotoxin) showing both ligands bound at the active site, as observed in the PLK D235A mutant structure. In all the structures Mg^{2+} is mediating interactions between the ATP γ-phosphate and the phosphate group of the other ligand. In addition, the interactions of the two phosphate groups with the anion hole of the protein help stabilize the complex.

Interestingly, unlike *E. coli* PLK, the homolog PLK2 is always purified with a mixture of PL and PLP tightly bound at the active site as described above [55]. These observations are consistent with the PLK2 significant reduced activity. A study by Schirch et al., showed that when *E. coli* cells were induced to overproduce B_6-dependent enzymes, there was an increase in PLP production that could be accounted for by the increased need of the synthesized apo-B_6 enzymes [72]. This shows that *E. coli* cells rapidly adapted to a significant need of PLP, but they only made enough to supply the requirement and the amount of free PLP did not increase. Quite significantly, there was no apparent increase in the activity of *E. coli* PLK, prompting the speculation that PLK2 may be the PLP donor. These results suggest that this homolog may be functioning in the B_6 salvage pathway, at least in prokaryotes to regulate the supply of PLP [55]. Recently, we have found that human PLK expressed in *E. coli* is purified as a complex with PLP. The stoichiometric amount of PLP bound to the enzyme increases from 20% to almost 100% if MgATP is used in the purification buffers (unpublished data). This bound PLP, in the presence of MgATP, is not removed by dialyses or chromatography, but is easily transferred to apo-B_6 enzymes. This feature is believed to have implications on both the regulation of PLK activity and on the delivery of free PLP to the requiring enzymes in the cell.

2.5. PLP deficiency due to drug-induced inhibition of PLK activity

Several drugs, as well as natural substances, are known to antagonize the action of vitamin B_6. Some classes of substances achieve these effects by inhibiting the activity of PLK, causing seizures, headache, agitation, convulsions, unconsciousness, paralysis and even death. The adverse effects caused by these compounds may be alleviated by co-administering vitamin B_6 supplements. Examples of compounds or drugs that have been identified to have an inhibitory effect on PLK are caffeine, theobromine, theophylline, ginkgotoxin, enprofylline, roscovitine, lamotrigine, isoniazid, levodopa, cycloserine and D-penicillamine [24,25,63,73–75]. The inhibiting drugs might be divided into three groups. The first group, including theophylline and progabide, inhibits PLK using either PL or PM as substrate and thereby might be considered true inhibitors; they do not form covalent complexes with PL or PLP. The second group, which includes cycloserine, dopamine, isoniazid and thiamphenicol glycinate, react with PL or PLP to form covalent complexes, and kinetic studies suggested that the observed PLK inhibition is due to these complexes. A third group, which consists of levodopa, D-penicillamine and muzolimine, inhibits PLK using PL, but not PM, as substrate; they do form chemical derivatives with PL or PLP, although these complexes do not seem to have a direct inhibitory effect on PLK [24]. The detailed mechanism of action of some of these compounds on PLK activity has been studied, the most potent inhibitors being theophylline with a reported K_i of 3 μM and ginkgotoxin with K_i of 0.4 μM [24,25]. Ginkgotoxin was actually shown to act as a substrate of PLK. Structural studies show both compounds to bind at the pyridoxal binding site ([25] and unpublished data).

3. Pyridoxine 5′-phosphate oxidase (PNPOx)

3.1. Characterization and catalytic function of PNPOx

Pyridoxine (pyridoxamine) 5′-phosphate oxidase (PNPOx) catalyzes the FMN-dependent oxidation of the 4′-hydroxyl group of PNP or the 4′-amino group of PMP into the aldehyde group of PLP (Fig. 5A) [42]. The enzyme is coded by a *pdxH* gene located, in humans, on chromosome 17q21.2. PNPOx has been purified and characterized from various

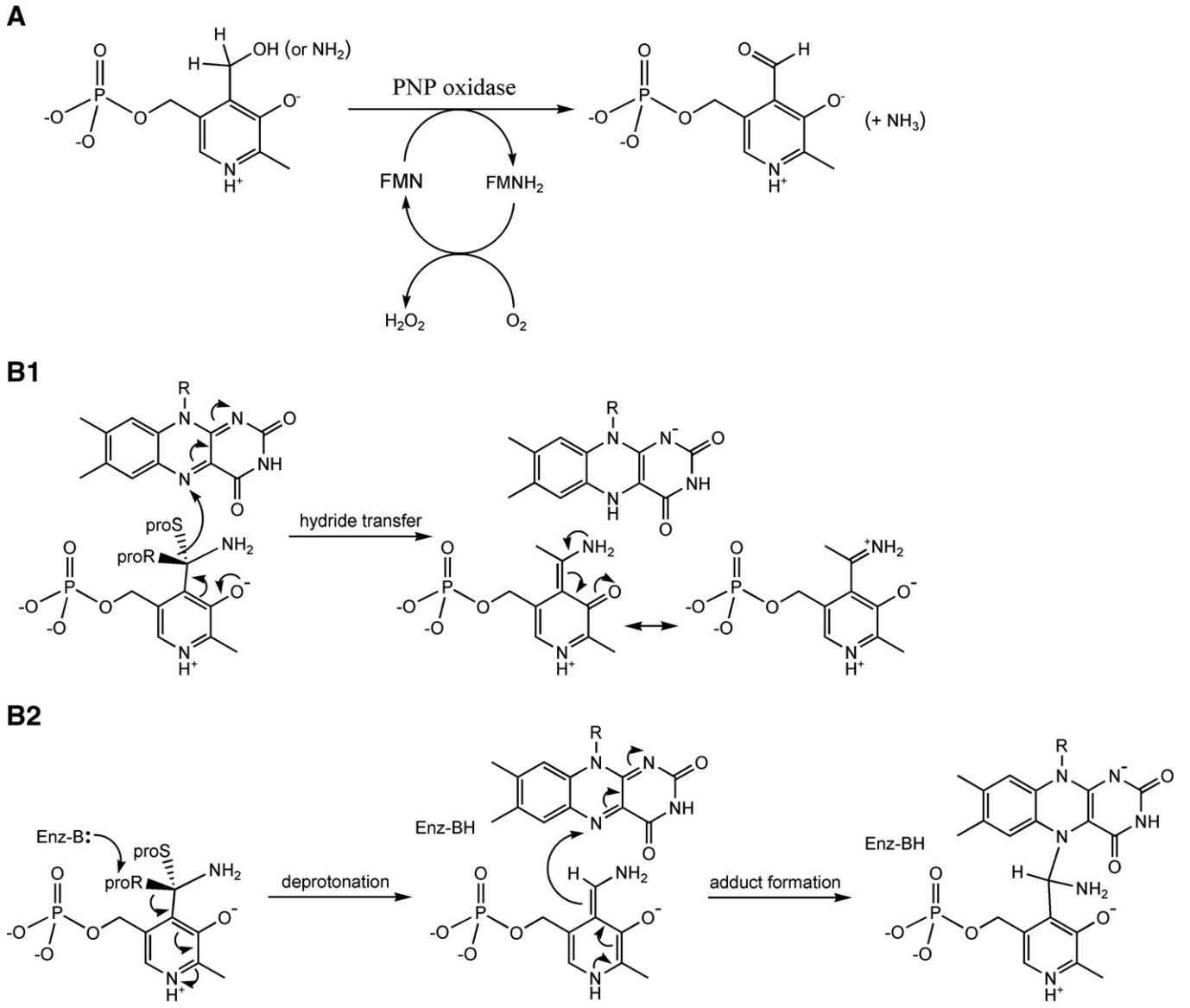

Fig. 5. (A) Reaction catalyzed by pyridoxine 5′-phosphate oxidase (PNPOx); (B) Description of two different mechanisms for the transfer of a pair of electrons from the C4′ of the substrate PMP (or PNP) to FMN in the reaction catalyzed by PNPOx. (B1) Possible mode of resonance stabilization during direct hydride transfer of C4′ *pro*R hydrogen of PMP to FMN. (B2) Removal of the *pro*R proton at C4′ of PMP to generate a carbanion; the carbanion then forms a covalent adduct with FMN as part of electron transfer process.

sources, including humans, sheep, rat, pig, rabbit, insect and *E. coli*, with the most extensive studies performed with the rabbit liver enzyme, and quite recently with both the human and *E. coli* enzymes [42,76–92]. PNPOx is the smallest member of the flavin-containing oxidase family, with the protein data bank listing several dozen enzymes. There is considerable sequence homology between PNPOx enzymes, and detailed sequence analysis has been reported [93].

Kinetic studies using rabbit PNPOx indicated that the reaction proceeds through a two-electron transfer, and has led to the proposal of two possible catalytic mechanisms for oxidizing PNP and PMP to PLP. The first is a direct hydride transfer from C4′ of the substrate to N^5 of FMN, to generate PLP and $FMNH_2$ (Fig. 5B1). The second possible mechanism involves the presence of a base at the active site that removes a proton from C4′ of the substrate, which then attacks FMN forming a covalent complex; collapse of this complex would generate PLP and $FMNH_2$ (Fig. 5B2). In either case, FMN is regenerated by the transfer of the two electrons to oxygen, forming hydrogen peroxide. Experiments utilizing site-specific mutants of the active site, isotope-labeled PMP, kinetic and structural studies have shown that the oxidation process involves direct hydride transfer from PNP or PMP to FMN. The *E. coli* enzyme was shown to be stereospecific for the abstraction of the *pro*R hydrogen atom on the C4′ of the substrate, in contrast with what was found for the rabbit liver PNPOx, in which lack of stereospecificity was shown [83,94]. Extensive characterization of both substrate and co-enzyme specificity has been performed. In addition to PNP and PMP, the enzyme can also use a number of substituted secondary amines of PMP as substrates [79,89]. The eukaryotic enzyme is equally efficient with both substrates, but the *E. coli* enzyme greatly favors PNP over PMP [42]. The K_m values for PNP and PMP are ~1.0 μM with the human enzyme, while with *E. coli* PNPOx, the K_m for PMP is 105 μM, 50-fold higher than for PNP (2.0 μM). The human and *E. coli* enzymes are sluggish, having a turnover of only ~0.20 s^{-1} with PNP and PMP [42,77,92].

Remarkably, various functional and structural studies have shown PNPOx to contain a non-catalytic site that tightly binds a second PLP molecule on each subunit. Even though this PLP remains bound during dialysis and size-exclusion chromatography, as for PLK the tightly bound PLP is readily transferred to apo-B_6-dependent enzymes, such as serine hydroxymethyltransferase (SHMT). In addition to serving as a conduit for delivery of PLP to the requiring

enzymes, the non-catalytic PLP binding site may play a role in PLP regulation [77,95,96].

3.2. Crystal structure of PNPOx

Our research group was the first to publish the crystal structure of PNOx, the first enzyme in both the biosynthetic and salvage pathways for which a structure was determined. Currently, the crystal structures of the human [77], *E. coli* [93,94,96], *M. tuberculosis* [97] and *D. vulgaris* [98] enzymes are known, and they all show a similar two-fold related dimer, with two FMN binding sites. Each monomer is made up of a two-domain α/β-barrel fold (Fig. 6A). The binding site of FMN, which is located in a deep cleft formed by the two subunits, is conserved in the PNPOx structures. The FMN makes extensive hydrogen bond interactions with highly conserved residues from both subunits [93]. PLP and PNP bind at the *re* face of FMN, with the phosphate group pointing out of the catalytic cavity mouth, in contrast to the FMN phosphate moiety that points downward into the cavity bottom. The substrate and the cofactor lie almost parallel, and the distance of 3.4 Å between the C4′ of PLP and the N^5 of FMN is optimal for the proposed hydride transfer (Fig. 6B).

The crystal structure of the unliganded enzyme shows an open active site [93]; however, binding of either PNP or PLP elicits a protein conformational change that partially closes the active site [96]. There are significant interactions between the FMN and PNP that serve to stabilize the latter. Interestingly, a monoclinic crystal form of the enzyme in complex with PLP shows previously unobserved N-terminal residues that fold over the active site to completely close it and sequester the ligand from the solvent. It has been suggested that this crystal structure may represent the catalytic state conformation [94].

An unanswered question is the location of the functionally observed non-catalytic tight binding PLP site. We have shown in

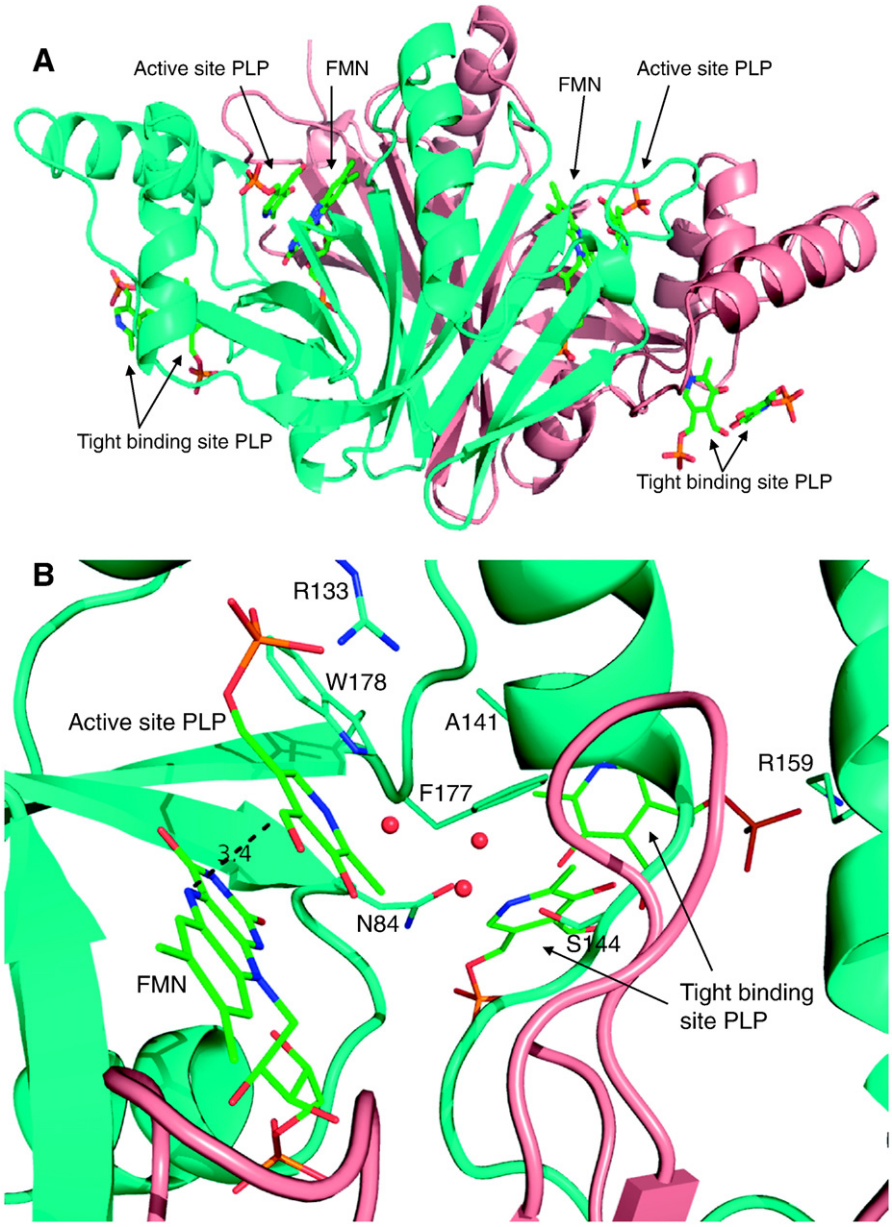

Fig. 6. (A) Ribbon diagram of the dimeric crystal structure of *E. coli* PNPOx complexed with PLP (PDB ID: 1G79). Monomers are in cyan and salmon. FMN and PLP from both active site and tight binding site are shown as sticks with atom based colors. (B) Close-up view of the same structure showing the putative tunnel between the active site and the non-catalytic PLP tight binding site. The residues lining the tunnel are shown as sticks. For clarity, not all residues are shown. Water molecules in the tunnel are shown as red spheres. In the active site, the distance in angstroms between C5′ of PLP and N^5 of FMN is shown. Figures were generated using MacPyMOL.

another crystal structure of the *E. coli* enzyme that a second PLP binds to PNPOx at ~11 Å from the active site, prompting speculation that this crystallographic secondary binding site corresponds to the functionally observed tight PLP binding site [96]. This second PLP molecule is seen in two different conformations, with 70% and 30% occupancy, respectively (Fig. 6A and B). The molecule with higher occupancy lies in a cavity formed by flexible loops at the domain interface. The plane of the pyridine ring is sandwiched between the side-chains of Phe177 and Lys145 (which also makes a hydrogen bond to the PLP phosphate moiety), and is located close to what appears to be a tunnel that leads to the active site (Fig. 6B). Adjacent to Phe177, and also guarding the tunnel is Asn84, which makes a close hydrogen bond to the pyridine nitrogen of PLP. The conformer with the lower occupancy makes fewer interactions with amino acid side-chains, namely a salt bridge with Lys159 and a side-to-face interaction with Phe177. The contact with Phe177 may be important because any conformational changes that involve Phe177 would help dislodge the PLP into the solvent. The putative tunnel between the two PLP binding sites is formed almost entirely by one subunit and it is mainly composed by glycine residues and small side-chains. There are structural water molecules located inside the tunnel. The shortest diameter of the tunnel, measured between Cα atoms, is about 8 Å. However, the presence of the side-chains of Trp178, Phe177, Arg133 and Asn84 in the cavity narrows the tunnel (Fig. 6B). Although the structures show the tunnel to be small for the passage of PLP, the channel could easily open up, since most of the surrounding protein structures are flexible. The possible role of the non-catalytic site in channeling a sequestered PLP to other enzymes is an important field of further investigations. Binding of PLP to the non-catalytic site of PNPOx might be one method for cells to regulate free PLP concentration *in vivo*. In *E. coli*, PNPOx is a relatively abundant enzyme [42], and there is enough enzyme to serve as a significant reservoir of PLP. Our group has also crystallized the human PNPOx with an excess concentration of PLP, and observed a density in a structural area which corresponds to the second PLP binding site of *E. coli* PNPOx (unpublished data). These structural data point to Asn84, Phe177 and Lys145 as being critical residues involved in binding the second PLP molecule, and prompt site-directed mutagenesis studies to check this hypothesis.

3.3. Regulatory mechanism

PNPOx-catalyzed reaction has been postulated to be one of the regulating steps in vitamin B_6 biosynthesis, since the product PLP strongly inhibits the enzyme. In both *E. coli* and mammalian PNPOx, PLP acts as a competitive inhibitor, exhibiting similar K_i values of ~5 μM [42,72,86]. Competitive substrate inhibition of the enzyme has also been observed using PNP as substrate, with a K_i of ~50 μM, and was partially relieved on increasing the O_2 concentration. In contrast, PMP does not seem to inhibit the oxidase activity [85].

3.4. Polymorphism in PNPOx and PLK

Severe deficiency of PLP in the cell can occur as a result of pathogenic mutations in the salvage enzymes, in particular in PNOx. One of the well characterized disease states attributed to PLP deficiency due to polymorphism in PNPOx is neonatal epileptic encephalopathy (NEE). NEE is a severe disorder which manifests a few hours after birth with intractable seizures usually unresponsive to conventional anticolvulsant treatment. Surviving children are usually mentally retarded and show an abnormal dependence on vitamin B_6 in the form of PLP [19]. At least five mutations in the gene of the oxidase, including homozygous missense (R95C, R95H, and R229W), stop codon (X262Q) or splice site (IVS3-1 g>a) mutations are known to be lethal resulting in NEE [21,99–101]. Our group has used site-directed mutagenesis, enzyme kinetics, X-ray crystallography and other functional studies to understand at a molecular level how the R95C and R229W mutations affect the enzymatic activity. The studies showed that, together with a reduced FMN affinity, the mutant enzymes had distorted active site geometries which resulted in a significant reduction of substrate affinity and catalytic activity; in the light of these results, it is plausible that the use of riboflavin (vitamin B_2) and PN, in conjunction with PL, may offer some improvement over the current treatment protocol of PL or PLP alone due to the apparent loss of FMN from the R229W variant [102].

There are several other neurological pathologies, not to mention non-neurological disorders, that are suspected to be due to mutations in either PNPOx or PLK encoding genes. Such mutations may result in defective enzymatic activity, low protein expression levels, and impaired regulation mechanisms causing PLP deficiency. The human genes encoding PNPOx and PLK are located on chromosomes 17q21.2 and 21q22.3, respectively. Individuals with chromosome 21 trisomy (Down syndrome) have been reported to display a significant alteration of B_6 metabolism [22]. Moreover, a recent study has reported a strong association between several polymorphisms in the PNP oxidase gene and schizophrenia in the Japanese population [23]; these patients have unusually high concentrations of homocysteine, which is known to be a risk factor for schizophrenia [103]. Both cystathionine β-synthase and cystathionine γ-lyase, which function in the transsulfuration pathway that converts homocysteine to cysteine, are PLP-dependent enzymes. Therapy with vitamin B_6 reduces homocysteine levels and appears to improve the symptoms experienced by chronic schizophrenic patients [23,104]. Another study found that children with autism spectrum show consistently higher concentrations of primary B_6 vitamers and significantly lower levels of PLP as compared with normal subjects [105]. This study concluded that the autistic patients probably have defective PLK that was unable to metabolize the primary vitamers into PLP. Several other neurological disorders, including seizures, attention deficit hyperactive disorder, Alzheimer disease, Parkinson disease, learning disability, and anxiety disorders, have also been associated with PLP deficiency [99,106–109].

Although there is no direct evidence linking some of these neurological disorders to mutations in either PLK or PNPOx, the genes coding for these enzymes are clearly candidates for mutational analysis in affected patients. If such an error is identified, studies could be performed to investigate the role of the mutation in the enzymatic activity and the associated phenotypes. This perhaps would point to the correct pharmacologic intervention.

4. Mechanism of PLP transfer to apo-B_6 enzymes

Although much work has been done on the mechanism and structure of B_6 enzymes, little is known on how PLP is supplied to meet their requirement in terms of cofactor. Free PLP availability in the cell is significantly limited by the high reactivity of its aldehyde group, forming aldimines with amino groups on non-B_6 enzymes and amino acids [72], as well as by dephosphorylation by phosphatases. Non-specific binding with non-B_6 protein has been implicated in the toxic effect of vitamin B_6. Free PLP is believed to react with apo-B_6 enzymes, but the free PLP concentration is not enough to meet the requirements of the many B_6 enzymes [110,111]. The low *in vivo* concentration of free PLP, as well as its highly reactive nature raises the intriguing question of how the cell supplies sufficient PLP, with high specificity, to the dozens of B_6 enzymes. The traditional proposed mechanism involves a release of PLP from either PNPOx or PLK into solution, where it finds its way to the active site of an apo-B_6 enzyme (Fig. 7B). An obvious problem with this mode of PLP transfer is potential PLP interactions with nucleophiles or dephosphorylation by phosphatases, which will significantly deplete the free PLP level available for the apo-B_6 enzymes. A second possible mechanism could be that cellular amino acids may be the specificity factor that targets

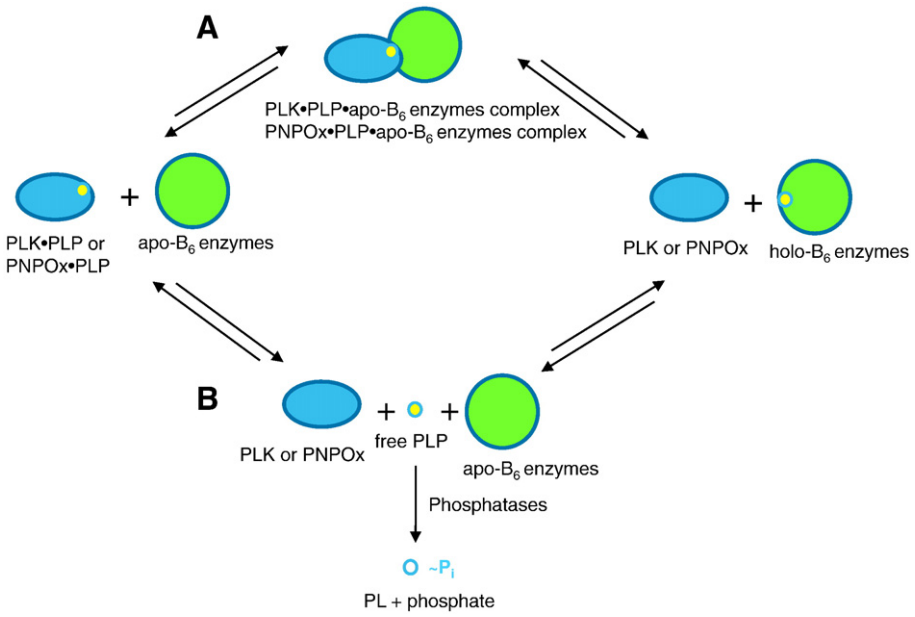

Fig. 7. Two possible mechanisms for the transfer of PLP from PLK or PNPOx to apo-B_6 enzymes. (A) Direct transfer mechanism (channeling); (B) free PLP mechanism.

PLP to the respective apo-enzymes. That is, each apo-B_6 enzyme may be converted to its holo form by reacting with the PLP-amino acid aldimines which have a similar structure to its substrate. This supposition is based on the fact that significant amounts of non-protein bound "free" PLP (non bound to proteins) is observed in *E. coli* cells [72]. It is suggested that most of this "free" PLP is bound to amino acids serving as a reservoir for free PLP which are made available to apo-B_6 enzyme when there is a need. A third alternative proposed mechanism for PLP transfer involves interactions between the donor enzymes (PLK and PNPOx) and acceptor B_6 enzymes, culminating in channeling of PLP from the former to the latter enzymes (Fig. 7A), thus avoiding the release of PLP into the solution. Although channeling is a controversial concept, it offers an efficient, exclusive, and protected means of delivery of the reactive PLP.

A study by Schirch's group showed that activation of apo-SHMT to the holo form in *E. coli* cell extracts was more efficient using a PNPOx•PLP complex (with PLP bound to the tight non-catalytic binding site) compared to the use of free PLP [95]. The authors suggested that free PLP in the extracts was forming non-productive aldimines with cell components, e.g. amino acids and proteins. In another study by Churchich's group, the investigators showed by using fluorescence spectroscopy, affinity chromatography and a trapping agent (alkaline phosphatases) that PLK forms a complex with aspartate aminotransferase (AAT) (K_d of 3 μM), and that the trapping agent did not inhibit the transfer of PLP to the PLP-dependent enzyme [111]. A recent study also using fluorescence polarization and surface plasmon resonance biosensor analyses showed that PLK can bind to alanine aminotransferase and glutamate decarboxylase with affinity constants in the low μM range [112]. These studies suggest that PLP is unlikely to be released into solution where it could be depleted, and channeling seems a strong possibility. However, a fascinating question regarding channeling remains an enigma and is yet to be resolved: how two very different donor enzymes are able to recognize and interact with dozens of acceptor enzymes that are characterized by at least five different folds? Nevertheless, there are examples of proteins that are known to interact with a large number of other proteins involving either separate and isolated sites or the same site [113].

The convergence of structural and functional data accumulated over the years, our ability to clone, express and purify PLK, PNPOx, and many B_6 enzymes, along with new evolving experimental technologies are now providing tools to successfully elucidate the mechanism of PLP transfer at several levels. An important observation made by our group is that both PNPOx and PLK bind PLP tightly and transfer it to apo-B_6 enzymes without apparent release of PLP into the solvent [77,95]; in several unpublished data, we have followed the activation of apo-SHMT or apo-AAT with free PLP or an equivalent amount of tightly bound PLP on PNPOX and PLK, in the presence and absence of PLP-phosphatase. Consistently with a channeling mechanism (Fig. 7A), we observed a severe inhibition of apo-B_6 enzyme activation to the holo form when free PLP was used, while the phosphatase (Fig. 7B) had no effect on the transfer of the PLP tightly bound to the salvage enzymes.

The channeling mechanism requires that a complex be formed between the oxidase or kinase and the B_6 enzymes, which has previously been observed by the Churchich and Cheung groups between PLK and several B_6 enzymes. We have also used flourescence polarimetry to study the binding interactions between *E. coli* PLK or PNPOx and several B_6 enzymes, including *E. coli* SHMT, AAT and L-threonine aldolase, all fold-type I enzymes, as well as with glycogen phosphorylase (a fold-type V enzyme). The results show that both PLK and PNPOx form specific interactions with every B_6 enzyme tested, with K_d ranging from 0.3 to 56 μM. In almost all cases, PNPOx exhibited a higher affinity than PLK in the corresponding interactions with the B_6 enzymes. The strongest affinity was between AAT and both salvage enzymes (~0.3 μM), while glycogen phosphorylase showed the weakest interaction, 56 μM with PLK and 25 μM with PNPOx. It is worth noticing that the most abundant B_6 enzyme in the body is indeed glycogen phosphorylase, which might explain its weakest interaction so that it does not outcompete other B_6 enzymes for PLP. Control experiments with several non-B_6 enzymes, including PLP-phosphatase, lysozyme, lactate dehydrogenase, and bovine serum albumin did not show any specific binding with either salvage enzyme. Affinity pull down chromatography experiments were also performed and confirmed the specificity of the interactions.

Although much work has been done in the field of PLP-dependent enzymes, the fundamental question of the availability of PLP in the cell has rarely been considered in terms of the mechanism and regulation of PLP homeostasis and in terms of transfer to newly folding B_6-enzymes. This new perspective may lead to novel and interesting findings in relation to the importance of vitamin B_6 in several cellular processes and in the onset of different pathologies.

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