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Intracellular Trafficking of the Pyridoxal Cofactor. Implications for Health and Metabolic Disease*

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Abstract

The importance of the vitamin B6-derived pyridoxal cofactor for human health has been established through more than 70 years of intensive biochemical research, revealing its fundamental roles in metabolism. B6 deficiency, resulting from nutritional limitation or impaired uptake from dietary sources, is associated with epilepsy, neuromuscular disease and neurodegeneration. Hereditary disorders of B6 processing are also known, and genetic defects in pathways involved in transport of B6 into the cell and its transformation to the pyridoxal-5'-phosphate enzyme cofactor can contribute to cardiovascular disease by interfering with homocysteine metabolism and the biosynthesis of vasomodulatory polyamines. Compared to the processes involved in cellular uptake and processing of the B6 vitamers, trafficking of the PLP cofactor across intracellular membranes is very poorly understood, even though the availability of PLP within subcellular compartments (particularly the mitochondrion) may have important health implications. The aim of this review is to concisely summarize the state of current knowledge of intracellular trafficking of PLP and to identify key directions for future research.

Keywords

Pyridoxal 5'-phosphate; vitamin B6; cofactor trafficking; mitochondria; iron homeostasis; heme biosynthesis

INTRODUCTION

The discovery of the B6 cofactor (pyridoxal 5'-phosphate, PLP) more than seventy years ago [1–3] initiated decades of fundamental biochemical research that has defined the unique role of that cofactor in enzyme catalysis [4–6], including activation of amine functional groups in metabolites [7,8] and, in the case of glycogen phosphorylase, activation of inorganic phosphate for phosphorolytic cleavage of glycosidic bonds [9]. PLP-dependent enzymes are

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now known to be an extraordinarily diverse family performing many essential metabolic functions in amino acid biosynthesis and catabolism, 1-carbon metabolism, membrane lipid biosynthesis, production of neurotransmitters and biogenic polyamines, as well as glycogen cycling and iron metabolism (iron-sulfur cluster and heme biosynthesis) (Table 1). While only PLP (and its congener, pyridoxamine 5'-phosphate (PMP)) can directly function in catalysis, the extended B6 family includes multiple vitamers forms, differing in phosphorylation state and modification of the 4' carbon (Figure 1).

The biosynthesis and metabolic interconversion of these vitamers has recently been worked out in detail, revealing two major biosynthetic pathways [10–13]. Vertebrates (including humans) lack both pathways, and thus rely on a salvage pathway to convert B6 provided by dietary sources or commensal intestinal flora into the enzyme cofactor, PLP. While not all organisms are able to synthesize the B6 cofactor, the ability to utilize environmental B6 through efficient uptake and salvage pathways appears to be universal [14]. Localization of these pathways has important implications for cellular metabolism. In particular, the exclusive localization of the salvage pathway to the cytoplasm of eukaryotic cells makes delivery of the membrane-impermeable PLP cofactor to enzymes in other subcellular compartments (particularly the mitochondrion) critically dependent on efficient mechanisms for *intra*-cellular cofactor trafficking. This requirement for intracellular PLP transport systems has been generally neglected and represents an important area for future research.

UPTAKE AT THE PLASMA MEMBRANE

The B6 story starts at the cell surface. Utilization of environmental sources of B6 is a universal feature of life, and specific transport systems exist for cellular uptake of B6 vitamers (Figure 2). In the yeast *Saccharomyces cerevisiae*, Tpn1p, a member of the purine-cytosine permease subfamily in the major facilitator superfamily, has been identified as the plasma membrane pyridoxine (vitamin B6) transporter [15,16]. Transport studies have demonstrated that Tpn1p is a unique, high affinity ($K_m = 0.55 \mu\text{M}$) pyridoxine carrier with broad substrate specificity for unphosphorylated B6 vitamers (including pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL)), utilizing a proton symport mechanism to concentrate B6 in the cell. Tpn1p is an integral membrane protein, predicted to be comprised of 12 transmembrane regions based on hydropathy analysis of the sequence.

Two homologous transport systems have been found in human cells [17–19], with distinct substrate specificity patterns. One system has been reported to be specific for unphosphorylated B6 vitamers (PN and PM) while the other may support uptake of the phosphorylated form (PLP) as well. Genetic identification and detailed characterization of these carriers is still lacking.

SALVAGE PATHWAYS

The capacity of a cell to utilize exogenous PN and PM entirely depends upon its ability to convert those vitamers to PLP through the salvage pathway (Figure 2)[14]. Phosphorylation of the 5' hydroxymethyl group by the broad-specificity pyridoxine (pyridoxal, pyridoxamine) kinase (PNK, yeast gene ID Bud17p)[14,20,21] efficiently traps the vitamer within the cell, since, in contrast to the precursor (PN), the charged product PNP is

membrane-impermeable. Trapping by phosphorylation is a general principle in cellular metabolism (“the importance of being charged” [22]) which also restricts the phosphorylated product to the subcellular compartment in which it is formed. Subsequent oxidation of the 4' hydroxymethyl group of PNP by pyridoxine oxidase (PNO, Pdx3p) [23] is required to form the functional cofactor.

Release of the PLP cofactor product from PNK appears to be very slow, leading to a suggestion that the enzyme may function as a chaperone [24] and the presence of a secondary PLP binding site on the surface of PNO [23] may reflect a chaperone role for that enzyme, as well. In solution, free PLP is subject to hydrolysis of the phosphoester bond (particularly at lower pH, or in the presence of phosphatases) and other reactions, including nonspecific imine formation by reaction with amine groups in amino acids and proteins. A PNK-PLP (or PNO-PLP) chaperone complex could suppress these side reactions and facilitate delivery of the cofactor to cognate apoenzymes for activation.

Formation of PLP (by *de novo* biosynthesis or salvage pathways) is a uniquely cytoplasmic function in the eukaryotic cell, since the enzymes involved in those pathways have an exclusively cytoplasmic localization. This localization pattern is consistent with the observation that most, but not all, of the PLP-dependent enzymes in the cell are also localized in the cytoplasm (Table 1). However, some PLP-dependent enzymes reside in other compartments, and, since PLP is membrane-impermeable, internal PLP trafficking pathways must be present to supply these enzymes with cofactor.

INTRACELLULAR TRAFFICKING

The presence of PLP-dependent enzymes in both the mitochondrion and peroxisome (Table 1) implies that there must be a mechanism for delivering the cofactor to enzymes in those compartments. Since the PLP cofactor is membrane-impermeable, membrane transport systems must exist to supply those two organelles with the PLP cofactor. Both compartments are bounded by bilayer membranes, but there are significant differences that make the two cases distinct.

The peroxisome is bounded by a single bilayer membrane, containing membrane-spanning pores (porin-like channels) that permit diffusion of small metabolites across the membrane [25–28]. These pores appear to mediate diffusional transport of a variety of small solutes [29–31] but it is unclear whether pores or specific carriers mediate the transport of cofactors (including coenzyme A, nucleotides and NAD⁺)[32–34]. The organization of the mitochondrion is more complex, with two concentric bilayer membranes [35,36]. The outer mitochondrial membrane (OMM), like the peroxisomal membrane, contains pores (VDACs) that allow free diffusion of molecules up to about 5 kDa [25–28], ensuring availability of cytoplasmic PLP to proteins in the mitochondrial intermembrane space (IMS). In contrast, the inner mitochondrial membrane (IMM) forms a strict permeability barrier preventing free diffusion of any molecules other than O₂ and CO₂.

MITOCHONDRIAL PLP TRANSPORT

Trafficking of B6 to the mitochondrion was first investigated more than 30 years ago, using radiotracer methods to monitor uptake of PN and PLP in isolated rat liver mitochondria [37,38]. Pyridoxine was found to permeate the mitochondrial membranes by simple diffusion, indicated by non-saturating uptake kinetics and equilibration across the membrane rather than concentration against a gradient. However, this diffusion process is unlikely to be biologically important, both because of the limited availability of free pyridoxine in the cell, and because of the lack of pyridoxine-processing salvage enzymes in the mitochondrial matrix, as described above.

A different type of transport behavior was observed for PLP, which was rapidly taken up by the isolated mitochondria. PLP first accumulated in the IMS, and subsequently entered the mitochondrial matrix in a concentrative process. Mitochondrial uptake of PLP was found to be “passive”. i.e., insensitive to inhibitors and uncouplers of oxidative phosphorylation, providing evidence for carrier-mediated PLP transport uncoupled from ATP synthesis. Surprisingly, this earlier work has not been extended by further studies.

The lack of additional studies may at least partly relate to the difficulty of mitochondrial PLP uptake measurements. The earlier experiments required challenging measurements of the [^{14}C]-PLP/ $^3\text{H}_2\text{O}$ ratio to demonstrate concentration of the cofactor, and fractionation experiments to define the distribution of the radionuclide in different mitochondrial compartments. In addition, PLP is a reactive aldehyde that can nonspecifically react with a variety of amines, complicating the interpretation of results.

IN ORGANELLO PLP TRANSPORT ASSAY

Innovative new approaches may be required to advance the field, and ultimately elucidate the mechanisms of mitochondrial PLP trafficking. In particular, molecular characterization of this essential cellular process may depend on availability of a simple and effective *in organello* assay for mitochondrial PLP transport, providing an alternative to the radiotracer experiments described above. The radiotracer methods used in the original studies of mitochondrial PLP transport are relatively challenging, involving complex assay protocols, and the reactivity of the PLP aldehyde towards protein lysine side chains and aminophospholipids can result in nonspecific binding, complicating analysis. It may be possible to address both issues by using a matrix-localized reporter protein to monitor membrane transport, as illustrated in Figure 3. Mitochondrial branched chain amino transferase (BCAT, Bat1p)[39,40] can be overexpressed in yeast mitochondria, which become loaded with high levels of the Bat1p apoprotein as protein import overwhelms PLP availability in that compartment (Figure 3, B&C). Mitochondria can be isolated by standard methods [41,42] and used for *in organello* uptake assays, fixing the localization of PLP at the endpoint by borohydride reduction of protein conjugates [43]. Affinity purification of tagged mBAT-TwinStrep (Figure 3B) then allows a direct read-out of the conjugated pyridoxine content by combined fluorescence and absorption measurements (Figure 3C). The results shown in Figure 3C indicate that overexpression of mBAT-TwinStrep protein in yeast mitochondria leads to accumulation of nearly pure apoprotein.

PLP-OME PROTEOMICS ANALYSIS

The identity of the molecular targets for PLP inside the mitochondrion is another interesting question with important biomedical implications. PLP transported into the mitochondrial matrix appears to be completely bound to protein, with no detectable free cofactor [38]. Since the distribution of the cofactor among protein complexes determines its metabolic effects, defining the PLP-linked proteome, the 'PLP-ome', may lead to deeper insight into the essential roles of the PLP cofactor in health and disease. The aim of a complete PLP-ome proteomics analysis would be to determine the distribution of PLP over the protein population, identifying binding sites and evaluating the degree of cofactor loading. Although the PLP cofactor is labile, PLP-protein adducts can be conveniently stabilized by borohydride reduction, converting the PLP imine (Schiff base) linkage to a hydrolytically stable amine [43]. The covalently-conjugated cofactor in the product remains attached through typical procedures for protein separation and analysis, allowing detailed characterization of the complex. A relatively simple solution to PLP-ome analysis that has already been described involves Western blot detection of PLP-protein conjugates following borohydride reduction using anti-pyridoxine (conjugated) antibodies, revealing the pattern of labeling over the entire protein population [43]. However, this method is fairly insensitive, does not provide a direct identification of the protein partner, and does not lend itself to a quantitative treatment of the data. Advanced methods of multiplexed mass spectrometry building on recent advances in phosphoproteomics analysis [44–48] are likely to be much more effective, providing information on cofactor loading and potentially defining the PLP binding site(s) within the protein.

Direct enzymatic analysis can also provide information on PLP binding reflected in the increase in catalytic activity following addition of the cofactor. This approach has been used to evaluate the degree of PLP loading for the mitochondrial matrix enzyme 5-aminolevulinate synthase (ALAS), which catalyzes a key step in heme biosynthesis (Figure 2), indicating that at least in some cases PLP availability limits the activity of mitochondrial enzymes [43]. Since the PLP cofactor can dissociate from protein complexes and redistribute on the timescale of enzyme assays, potential sources of the cofactor in the assay mixture need to be carefully controlled.

CARRIER ANALYSIS: *IN VITRO* ASSAY OF PLP TRANSPORT

The evidence for a specific transport pathway for mitochondrial uptake of PLP implies existence of a carrier that may be isolated for detailed molecular characterization. Over the past decades, intensive research has elucidated the mechanisms of mitochondrial transport, and the important role of mitochondrial carrier proteins (MCPs) in these processes [49–53]. The yeast MCP superfamily includes 35 members, including many whose functions have been assigned by genetic analysis or direct assays. A PLP carrier function has recently been proposed for a yeast MCP (Mtm1p) based on the knock-out phenotype and direct analysis of cofactor binding [43,54]. The purified recombinant protein tightly binds PLP ($K_D = 0.6 \mu\text{M}$ (fluorescence titration); $K_{D,1} = 2 \mu\text{M}$, $K_{D,2} = 47 \mu\text{M}$ (isothermal titration calorimetry, ITC)) while no binding of Mn^{+2} , methionine, cysteine or glutathione could be detected by ITC (unpublished results).

Direct evidence for a PLP transport function for Mtm1p (or another carrier) will require an effective *in vitro* transport assay. As described above, the design of an effective PLP transport assay is complicated by the reactivity of the 4' aldehyde, since transport across the membrane needs to be distinguished from nonspecific attachment of the cofactor to proteins or aminophospholipids on the outer surface of a membrane system, a problem that is not usually encountered in membrane carrier *in vitro* assays.

An approach to PLP membrane transport measurements based on catalytic amplification of the permeation signal through activation of an encapsulated PLP-dependent apoenzyme is shown in Figure 4. This hypothetical scheme utilizes a coupled assay to detect PLP in the lumen of a membrane-bounded vesicle and to monitor the progress of cofactor transport, using established membrane reconstitution and encapsulation methods [55–58]. While in principle, any PLP-dependent enzyme would be suitable, practical implementation of this type of assay will depend on both availability and stability of the apoenzyme, and availability of a suitable detection substrate. *Escherichia coli* tryptophanase (TnaAp, [EC 4.1.99.1])[59] appears to be a likely prospect for this application, since it can be prepared in large quantities as a 6×His tagged recombinant protein, can be rigorously converted to pure apoenzyme by dialysis against 1 M amino acid solution (e.g., D,L-alanine), and is highly stable as the apoprotein. A chromogenic substrate for TnaAp has been reported [60], as well as a fluorogenic substrate [61] which would greatly enhance the sensitivity of PLP detection.

PLP TRAFFICKING DEFECTS AND METABOLIC DISEASE

A number of important mitochondrial pathways, including those involved in amino acid biosynthesis, energy metabolism and metal homeostasis, require PLP for at least one step (Table 1, Figure 2). While there is some redundancy in pathways for amino acid processing between the cytoplasm and mitochondrial matrix (e.g., distinct cytoplasmic and mitochondrial isozymes for amino acid biosynthesis (Aat1p, Aat2p), catabolism (Bat1p, Bat2p), and C-1 metabolism (Shm1p, Shm2p)), the mitochondrial requirement for matrix PLP to support heme biosynthesis and Fe-S cluster biogenesis appears to be absolute, and may represent a critical feature linking PLP trafficking to health and disease. The connection between PLP availability and health is well-known [62–64], but the focus has generally been on dietary availability of the cofactor and cytoplasmic metabolism. Intracellular trafficking of PLP is clearly an essential function of the eukaryotic cell, one which has largely neglected, in spite of its fundamental biological importance. There is strong evidence linking defects in mitochondrial metabolite trafficking to metabolic dysfunction and human disease [65–67] indicating the importance of this area for future advances in cardiovascular health and understanding of neurodegenerative disease.

CONCLUSION

Enzymes that require the PLP cofactor are generally localized to the cytoplasm in eukaryotic cells, where conversion of the pyridoxine precursor occurs through the action of pyridoxine kinase and pyridoxine 5' phosphate oxidase enzymes. The unique localization of the latter two enzymes to the cytoplasm means that any PLP-dependent enzymes residing in other compartments (including the mitochondrion) must acquire the cofactor through a

mechanism involving transport of fully formed PLP, rendered impermeable to biological membranes by its charge and hydrophilicity. The clear link between mitochondrial PLP transport and essential pathways of metabolism, including heme biosynthesis and Fe-S cluster biogenesis (Figure 2), demonstrates the importance of this process for cell function. These crucial metabolic links may be revealed in hereditary disorders of PLP trafficking, or provide novel targets for drug development.

Abbreviations

MCP	mitochondrial carrier protein
PLP	pyridoxal 5'-phosphate
PN	pyridoxine
PL	pyridoxal
PM	pyridoxamine
PMP	pyridoxamine 5'-phosphate
ALAS	5-aminolevulinate synthase
ITC	isothermal titration calorimetry

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RESEARCH HIGHLIGHTS

- Intracellular trafficking of the PLP cofactor is an essential biological function
- Mitochondrial trafficking of PLP is required for heme and Fe-S cluster biosynthesis
- Transport systems for intracellular trafficking of PLP are largely undefined
- New approaches are required to investigate PLP trafficking mechanisms

Figure 1.

Structures of B6 vitamers. PLP, pyridoxal 5'-phosphate, indicating the numbering scheme for the B6 ring system; (1) pyridoxine (PN); (2) pyridoxine 5'-phosphate (PNP); (3) pyridoxamine (PM); (4) pyridoxamine 5'-phosphate (PMP); (5) pyridoxal (PL).

Figure 2.

Pathways for B6 transport and processing in eukaryotic cells. Uptake of pyridoxine at the plasma membrane allows conversion to PLP within the cytoplasm. Subsequent trafficking of the pre-formed PLP cofactor to the mitochondrion is required for essential processes of heme biosynthesis (Left) and Fe-S cluster biogenesis (Right). Yeast (*Saccharomyces cerevisiae*) gene IDs are used to identify specific elements of these pathways.

Figure 3.

Loading yeast mitochondria with recombinant Bat1p for *in organello* assay of PLP transport. (A) Assay scheme: yeast containing an expression vector encoding the mitochondrial branched chain amino transferase (Bat1) fused to a C-terminal TwinStrep affinity tag (pYES2mBAT-TwinStrep) are induced overnight (2% galactose, 2% lactate, 0.2% raffinose) and mitochondria isolated by subcellular fractionation (homogenization, differential centrifugation). Isolated mitochondria are suspended in isosmotic buffer and treated with PLP with rotisserie agitation. A timecourse may be generated by fixing the PLP bound to protein using borohydride reduction at the endpoint. StrepTactin affinity purification of the mBAT-StrepTag protein followed by fluorescence analysis permits quantitation of cofactor content of the protein. (B) Purification of recombinant mBAT-

TwinStrep protein from isolated yeast mitochondria. (1) intact isolated mitochondria, showing high level expression of recombinant mBAT-TwinStrep protein; (2) StrepTactin flow-through; (3) StrepTactin wash; (4–6) StrepTactin elution with desthiobiotin. Arrows (►) identify the location of the recombinant mBAT-TwinStrep protein. (C) Fluorescence spectra for affinity-purified mBAT-TwinStrep isolated from yeast mitochondria before (1) or after (2) incubation with PLP followed by reductive trapping with borohydride in the presence of 1 mM PMSF. Both samples 35 µg/mL protein in 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA.

Figure 4.

Hypothetical *in vitro* assay for PLP transport based on activation of an encapsulated apoenzyme. (Top) Purified carrier in proteoliposome is combined with giant unilamellar vesicles (GUV) containing encapsulated recombinant *E. coli* TnaAp (**E**) and a fluorogenic substrate (**S**). (B) Fusion of the proteoliposomes with the GUVs. (C) Transport of exogenous PLP leads to activation of TnaAp allowing fluorometric monitoring of PLP uptake.

Table 1Subcellular localization of pyridoxal 5'-phosphate dependent enzymes in *Saccharomyces cerevisiae*.

Enzyme Name	Abbreviation	EC No.	Gene ID ^a	Genetic Locus ^a
1. Cytoplasm				
Aspartate aminotransferase	AAS	2.6.1.1	Aat2	YLR027C
Aromatic aminotransferase I	ArAT	2.6.1.57	Aro8	YGL202W
Aromatic aminotransferase II	ArAT	2.6.1.57	Aro9	YHR137W
7,8-Diamino-pelargonic acid aminotransferase	DAPA	2.6.1.62	Bio3	YNR058W
Kynurenine aminotransferase	KAT	2.6.1.7	Bna3	YJL060W
Kynureninase	KYNU	3.7.1.3	Bna5	YLR231C
L-ornithine transaminase	OAT	2.6.1.13	Car2	YLR438W
Cystathionine γ -lyase	CTH	4.4.1.1	Cys3	YAL012W
Cystathionine β -synthase	CBS	4.2.1.22	Cys4	YGR155W
Dihydrosphingosine phosphate lyase		4.1.2.27	Dpl1	YDR294C
Glutamate decarboxylase	GAD	4.1.1.15	Gad1	YMR250W
Glycogen phosphorylase	GP	2.4.1.1	Gph1	YPR160W
Histidinol-phosphate aminotransferase		2.6.1.9	His5	YIL116W
Cysteine-S-conjugate β -lyase		4.4.1.13	Irc7	YFR055W
Serine palmitoyltransferase		2.3.1.50	Lcb1	YMR296C
Serine palmitoyltransferase		2.3.1.50	Lcb2	YDR062W
Bifunctional cysteine synthase/O-acetylhomoserine aminocarboxypropyltransferase		2.5.1.47	Met17	YLR303W
3-Phosphoserine aminotransferase	PSAT	2.6.1.52	Ser1	YOR184W
Serine hydroxymethyltransferase	SHT	2.1.2.1	Shm2	YLR058C
Branched-chain amino acid aminotransferase	BCAT	2.6.1.42	Bat2	YJR148W
γ -aminobutyrate (GABA) transaminase		2.6.1.19	Uga1	YGR019W
2-Aminoacidipate transaminase	AadAT	2.6.1.39		YER152C
Ornithine decarboxylase	ODC	4.1.1.17	Spe1	YKL184W
S-adenosylmethionine decarboxylase	AdoMetDC	4.1.1.50	Spe2	YOL052C
Phosphatidylserine decarboxylase	PSD	4.1.1.65	Psd1	YNL169C
2. Mitochondria				
Aspartate aminotransferase	AAT	2.6.1.1	Aat1	YKL106W
Alanine:glyoxylate aminotransferase	AGAT	2.6.1.44	Agx1	YFL030W
Alanine transaminase	ALT	2.6.1.2	Alt1	YLR089C
Acetylornithine aminotransferase		2.6.1.11	Arg8	YOL140W
Kynurenine aminotransferase	KAT	2.6.1.7	Bna3	YJL060W
L-serine (L-threonine) deaminase (catabolic)		4.3.1.19	Cha1	YCL064C
Glycine decarboxylase complex	GCC	2.1.2.10	Gcv2	YMR189W
5-Aminolevulinate synthase	ALAS	2.3.1.37	Hem1	YDR232W
Threonine deaminase	TD	4.3.1.19	Ilv1	YER086W

Enzyme Name	Abbreviation	EC No.	Gene ID ^a	Genetic Locus ^a
Cysteine desulfurase		2.8.1.7	Nfs1	YCL017C
Serine hydroxymethyltransferase	SHT	2.1.2.1	Shm1	YBR263W
Branched-chain amino acid aminotransferase	BCAT	2.6.1.42	Bat1	YHR208W
3. Peroxisome				
Alanine:glyoxylate aminotransferase	AGAT	2.6.1.44	Agx1	YFL030W

^a Gene ID and genetic locus from *Saccharomyces cerevisiae* genome data.