Investigations into the Biosynthesis of Vitamin B6 and its Role in Developmental Processes And Stress Response in Plants

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Vitamins are defined as organic micronutrients that must be obtained in the human diet and are essential compounds for the survival of all organisms. Plants are critical sources of these compounds. However, despite this recognition, the importance of these compounds to plants themselves has been somewhat overlooked. Thus, in most cases the physiological, biochemical and molecular mechanisms that contribute to their synthesis, transport and accumulation in plants are not known. This is especially the case for the water-soluble B vitamins. The main focus of my research is on vitamin B1 (thiamin) and vitamin B6 (pyridoxine). The essentiality of these compounds to all organisms is recognized by their well-established function as coenzymes for numerous metabolic enzymes. Vitamin B1 is necessary for the catalytic activity of enzymes of the citric acid cycle, glycolysis and the pentose phosphate pathway, in addition to amino acid and isoprenoid biosynthesis. Deficiency is a widespread health problem particularly in countries where rice is a major constituent of the diet, since grain polishing removes most of the thiamin in the bran. Therefore, it is of major interest to define the pathway of biosynthesis, a virtually unexplored area in plants, which may assist in the overproduction of the vitamin for beneficial purposes. On the other hand, vitamin B6 is renowned as being involved in more functions than any other single nutrient being a cofactor for a diverse array of enzymes ranging from amino acid metabolism to antibiotic biosynthesis. In plants, it is also used in many secondary metabolite pathways, *e.g.* alkaloid biosynthesis. More recently, some novel functions have been revealed for both of these vitamins that go beyond their role as cofactors. Vitamin B6 has been shown to be an antioxidant with potency equivalent to those of vitamins C and E, and has been implicated in alleviating oxidative, UV and salt stress in plants, while vitamin B1 has been linked to systemic acquired resistance in plants, in addition to DNA damage tolerance. The involvement of vitamins B1 and B6 in stress responses was completely unprecedented and may be of economical importance in the development of stress-tolerant crops. Furthermore, as the biosynthesis pathways exist in only bacteria, fungi and plants, *i.e.* being absent from humans, they may provide novel drug targets. Therefore, the aims of this research are to establish the pathways of biosynthesis, decipher how their production is regulated, dissect modes of transport, assess effects of over- or

under- accumulation and unravel novel roles of vitamins B1 and B6 and their mechanisms. An interdisciplinary approach is taken combining molecular cellular biology, biochemical, biophysical, and physiological methods. The studies are predominantly carried out in the plant model, *Arabidopsis thaliana*, but bacterial models are also utilized to decipher data rapidly. While these issues are of fundamental importance and are of broad interest to the scientific community, the answers to these questions may permit either the production of these compounds for beneficial effects or their depletion in the context of herbicide or antibiotic development.

Vitamin B6 biosynthesis

To date, my work has primarily focused on vitamin B6. We have been instrumental in unraveling a novel pathway of vitamin B6 biosynthesis that exists in the majority of organisms that can synthesize this compound. We have demonstrated that the vitamin can be synthesized from glycolytic and pentose phosphate pathway intermediates, instead of deoxyxylulose phosphate (DXP) and 4-phosphohydroxythreonine, as is the case in the well-characterized pathway of *E. coli*. We coined the term "DXP-dependent" and "DXP-independent pathway" to distinguish the two routes. We showed that the two proteins (PDX1 and PDX2) involved in the DXP-independent pathway work together as a glutamine amidotransferase, with PDX1 as the synthase domain and PDX2 as the glutaminase domain and remarkably result in the direct synthesis of the cofactor vitamer, pyridoxal 5′-phosphate (PLP). The PDX1 and PDX2 proteins together are now known as PLP synthase. The activities of each were reconstituted with the proteins from bacterial and plant models as well as the malaria parasite, *Plasmodium falciparum*. We use bacteria as a model to decipher data rapidly that can then be applied to the more time-consuming plant model, whereas *P. falciparum* was chosen in the context of this pathway being a potential drug target due to its absence in animals. In each case, the proteins were shown to be dependent on each other for activity, and a molecular explanation for this became clear when we were able to solve, not only the structure of the individual proteins, but also that of the complex between the two entities from the Gram-positive bacterium, *B.*

subtilis (in collaboration with Dr. Ivo Tews, University of Heidelberg). PDX1 itself is an $(\beta\alpha)_8$ barrel, but has a unique quaternary structure consisting of twelve protein units assembled as two opposing hexameric rings (Figure 1). PDX2 on the other hand is monomeric and displays typical Rossmann topology, *i.e.* a classic mixed α/β three-layer sandwich with a seven-stranded twisted mixed parallel β-sheet flanked by six α-helices on the N-terminal stretch of the sheet. While it was gratifying to know the individual structures, the major breakthrough came with solving the PDX1/PDX2 protein complex with the PDX2 substrate glutamine bound in its active site. The PLP synthase complex resembles a cogwheel in which every PDX1 subunit of the double hexameric ring binds a discrete PDX2 subunit (Figure 1). This architecture is unique within the glutamine amidotransferase family and comparison of the individual subunits with that of the macromolecular assembly revealed a novel mode of glutaminase and synthase interaction. An α-helix at the N-terminus of PDX1, named αN, proved to be vital for the interaction of the two proteins and furthermore for activation of the glutaminase activity of PDX2.

In addition to the ornate architecture, PLP synthase is a unique enzyme in nature with an exceptional polymorphic synthetic ability. To date, the co-crystallization of buffer components allowed active site residues to be proposed, some of which have been confirmed by site directed mutagenesis and enzyme assays.

A hypothetical mechanism of action was first proposed by Begley and colleagues in 2005. Since then, in collaboration with Prof. Duilio Arigoni (ETH Zürich), we have been able to demonstrate some key features along the catalytic path, in particular many of the early steps in catalysis, resulting in the necessity to revise the previously proposed mechanism. One key component was the detection of a covalent chromophoric reaction intermediate that occurs after dephosphorylation of the pent(ul)ose phosphate substrate and is dependent on glutamine hydrolysis by PDX2. The later steps of the mechanism now need to be unraveled and include the nature of the triose binding site as well as the reason for the curious observation of a second phosphate binding site between the two hexameric rings in PDX1. Moreover, other reac-

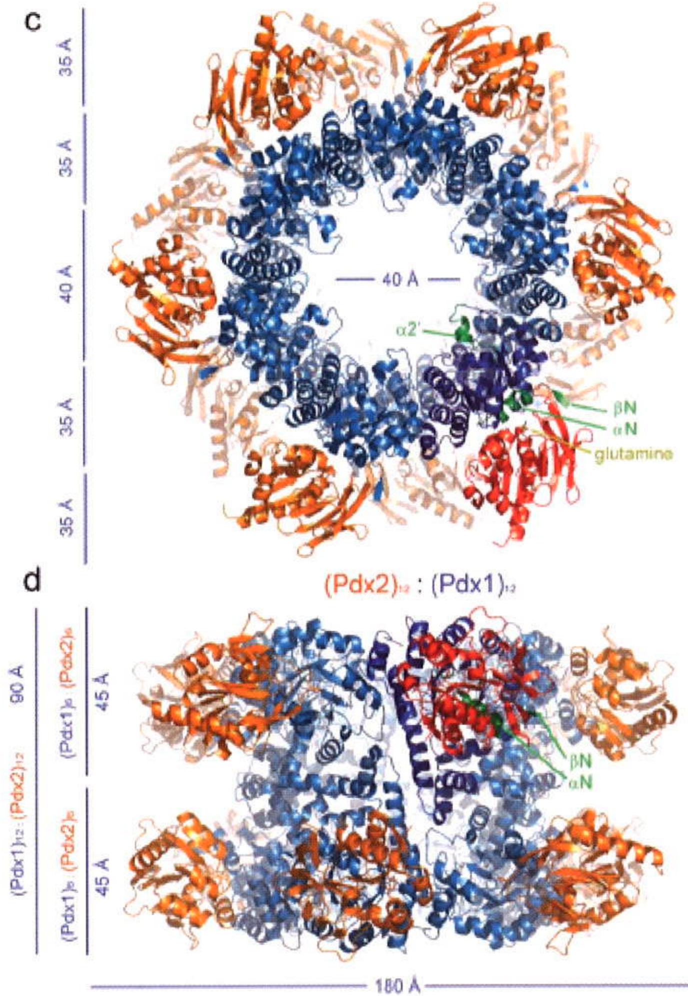

Figure 1: Architecture of the PLP synthase complex from *B. subtilis*, shown in two orientations (top and side view) rotated by 90°. Twelve PDX2 glutaminase subunits (orange) attach to a double-ring-like core formed by twelve PDX1 subunits (blue). A single heterodimer is highlighted in dark blue (PDX1) and red (PDX2). In PDX1, the N-terminal β-strand, βN, and α-helix, αN, as well as helix 2', which by comparison to the autonomous PDX1 structure, form upon complex formation are highlighted in green. Approximate sizes are given in Ångstroms (10^{-10} m).

tion intermediates need to be identified to validate existing mechanistic proposals.

The study of the PDX1 and PDX2 proteins from different organisms has greatly assisted us in taking this field forward, with the occurrence of the proteins in Arabidopsis being a particularly interesting one. There are three homologs of *PDX1* in the haploid genome of Arabidopsis (which we named *PDX1.1*, *PDX1.2* and *PDX1.3*), but only two are able to synthesize vitamin B6 (*PDX1.1* and *PDX1.3*); there is only a single gene coding for *PDX2*. The analysis of individual and double knockout mutants for the functional proteins allowed us to demonstrate the essentiality of the PDX proteins for growth and development in Arabidopsis. Knocking out either *PDX2* alone, or *PDX1.1* and *PDX1.3* together results in an embryo-lethal phenotype (Figure 2). Interestingly, an expression analysis revealed that both PDX1.1 and PDX1.3 predominantly accumulate in shoot rather than root tissue, with PDX1.3 being more abundant than PDX1.1. Curiously though, the *pdx1.3* individual knockout mutant displays a severe impairment in root growth not seen in the *pdx1.1* mutant (Figure 2) and awaits further investigation.

The discovery of the DXP-independent pathway in plants permitted the demonstration by us and others that vitamin B6 is not only essential for plant growth and development, but also in the response to salt, osmotic and oxidative stress. Plants must regulate the biosynthesis of vitamin B6 to fulfill metabolic requirements in specific tissues and developmental conditions; this can be either as a cofactor, an antioxidant or perhaps for thiamin biosynthesis. Interestingly, preliminary analyses of transcript abundance have shown that the expression of the *PDX* genes is differentially modulated by developmental cues and by environmental signals such as light, UV, cold and salinity, in addition to plant pathogen defense responses. Furthermore, our current analyses indicate that various abiotic stress treatments lead to an alteration of the overall vitamin B6 content. It should be recognized that vitamin B6 is a general term for pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives. While it is well established that pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP)

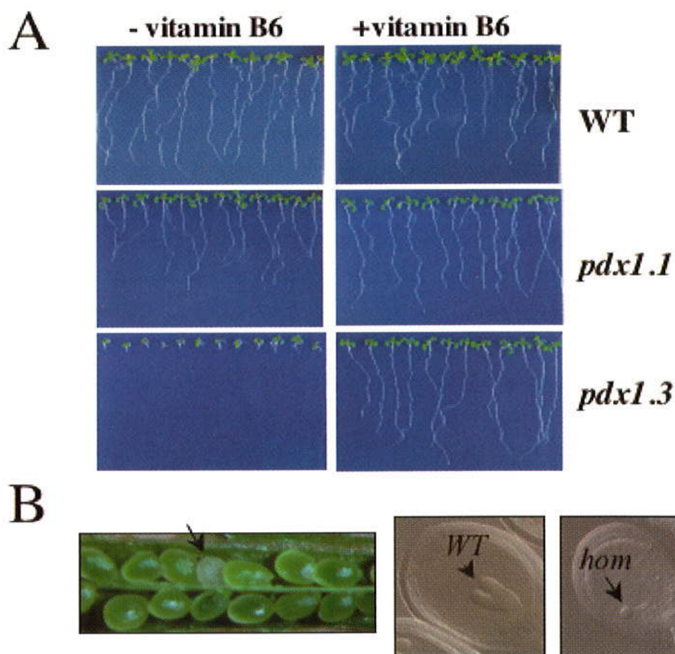

Figure 2: Phenotype of PDX1 knockout mutants. **(A)** Root growth of WT, *pdx1.1* and *pdx1.3* in the presence and absence of vitamin B6. The severe growth impairment of *pdx1.3* is rescued in the presence of the vitamin. **(B)** Left side: Immature silique of a heterozygous double knockout of both *pdx1.1* and *pdx1.3*. One sixteenth of the seeds were albino. Right side: Cleared whole mounted WT and albino seeds showing arrest of *pdx1.1/pdx1.1 pdx1.3/pdx1.3* at the globular stage of embryo development. Note *pdx2* is arrested at the same stage.

are the cofactor forms, it is not known which vitamers are involved in the antioxidant function, nor are the level and compartmentation of the vitamers known. The *de novo* biosynthesis pathway results in the formation of the cofactor form, PLP, but there is also a salvage pathway in place in most organisms through which the different vitamers can be interconverted. This pathway comprises pyridoxine 5´-phosphate oxidase (PDX3), which is active on both PMP and PNP converting them to PLP; pyridoxal kinase (PDXK), which catalyzes the conversion of PN, PL or PM to the respective 5´-phosphate ester, and in some organisms,

an apparently specific PL kinase is active. We have identified homologs of many of the salvage pathway genes in Arabidopsis and have established a HPLC assay to detect all six vitamers, and are therefore in a position to address the importance of each vitamer.

Vitamin B1 metabolism

Despite the importance of vitamin B1, its metabolism is a very poorly researched area in plants. We have begun to investigate its biosynthesis in Arabidopsis with a particular focus on the biosynthesis of the pyrimidine heterocycle, which could either be derived from AIR as in bacteria, or from vitamin B6 as in yeast, or indeed both. Specifically, we have investigated the single homolog of the THIC protein in Arabidopsis, which had not yet been characterized. This protein appears to be essential for growth in Arabidopsis as a severe knockdown line has a seedling lethal phenotype that can be rescued by thiamin supplementation, suggesting that THIC is a major player in vitamin B1 biosynthesis in plants. Moreover, we have been able to heterologously express the protein in bacteria and can obtain the protein in a remarkably high yield. This enables us to carry out a full biochemical characterization. So far, we have been interested in defining factors necessary for activity and have very strong evidence for the presence of an iron-sulfur (Fe-S) cluster in the protein. The conclusion is based on the UV-visible spectrum of the purified protein and analysis of Arabidopsis mutant plants deficient in NifS, a cysteine desulfurase necessary for Fe-S generation in plants. Furthermore, we have identified a conserved motif that may classify THIC as a member of the family of proteins dependent on the 5′-deoxyadenosyl radical generated from *S*-adenosyl methionine. These facts will greatly assist in deciphering the mechanism of action of this intriguing enzyme. In addition, we have evidence that the enzyme is a key regulatory protein within the vitamin B1 biosynthesis pathway as the level of its mRNA appears to be very strongly regulated by thiamin in the growth medium.

From the beginning of 2009, I will take up the position as associate professor of Plant Biochemistry at the University of Geneva, where I will continue to explore these research areas. I would like to thank the

Latsis Foundation for this encouraging award and in particular my mentor at the ETH Zürich, Prof. Nikolaus Amrhein, whose support and encouragement over the years while I was a post-doc and later group leader made all of this research possible. I would also like to thank all of my co-workers and colleagues whose contributions were also fundamental for the success of this research area.