Università degli Studi di Parma
Department of Chemistry, Life Sciences and Environmental Sustainability
Laboratories of Biochemistry and Molecular Biology
PhD Course in Biotechnology and Life Science
XXXIV Cycle

RESEARCH ACTIVITY

"Biochemical studies of pyridoxal-5'-phosphate-dependent enzymes with known and unknown function."

During this first year of PhD I worked at of two different projects that have in common a focus on pyridoxal-5'-phosphate (PLP)-dependent enzymes. On one hand I analyzed the reaction of the mouse ornithine aminotransferase (mOAT) through a continuous coupled assay with a *E. coli* pyrroline-5-carboxylate reductase (ecPYCR1), focusing on the cyclization rate of L-glutamate semialdehyde (GSA) to investigate if it requires enzymatic catalysis *in vivo* by its cyclization rate. On the other hand I carried out a bioinformatical identification and a preliminary biochemical characterization of a bacterial PLP-dependent enzyme presumably involved in aminophosphonate biodegradation.

Mammalian ornithine aminotransferase is a mitochondrial enzyme whose deficit causes gyrate atrophy, a rare hereditable dystrophy of the retinal that leads to total blindness. This severe disease suggests that OAT plays an important role in metabolism. OAT is a PLP-dependent aminotransferase that catalyzes, through a ping-pong mechanism, the transfer of δ-amino group of L-ornithine to α-ketoglutarate, leading to the production of GSA and L-glutamate. GSA, that which containing both an aldehyde and an amine group, can spontaneously form pyrroline-5-carboxylate (P5C) that is substrate of PYCR1, a NADH/NADPH-dependent enzyme that catalyzes this last step of L-proline biosynthesis. If the spontaneous cyclization rate of GSA is a slow step, it could require catalysis *in vivo*. To investigate this hypothesis, I set up a continuous coupled assay with mOAT and ecPYCR1: as P5C is formed, it is reduced to L-proline by ecPYCR1, with the simultaneous oxidation of NADPH to NADP+ that can be monitored spectrophotometrically by the decrease in absorbance at 340 nm. Usually, a slow intermediate step in a coupled assay should appear as a slow-starting kinetics, in which duration of the delay will depend on the rate of the spontaneous reaction. To confirm that this approach works I set up a further enzyme assay monitoring the reaction of *E. coli* threonine dehydratase (ecTD), another PLP-dependent enzyme, with L-serine through the lactate dehydrogenase (LDH)-coupled assay. The product of ecTD is 2-aminoacrylate, which spontaneously react with water to yield ammonia and pyruvate, which in turn is a LDH substrate. The spontaneous hydrolysis of 2-aminoacrylate might take a few seconds, resulting in a small but appreciable lag in the kinetics of the coupled assay. However, the lag can be eliminated by adding to the reaction mixture the 2-iminopropanoate deaminase that accelerates the hydrolysis step. Different reaction conditions were tested to maximize the rate of the mOAT reaction while possibly slowing the spontaneous cyclization of GSA, and some slow-starting kinetics were observed. Slow-starting kinetics need to be interpreted correctly because they could hint to some interesting properties of the enzyme but also to some possible artifacts. For example, an inadequate amounts of indicator enzyme in a coupled assay typically leads to an initial lag phase, which disappears upon increasing the amount of the indicator enzyme present; and I also observed an artifactual slow-starting kinetics at low pH, attributable to relief of substrate inhibition by α-ketoglutarate: in this case the activity rises as substrate concentration decreases, up to a maximum value attained at some 'optimal' substrate concentration. After ruling out artifacts and other possible causes, I didn't notice any lag due to a slow GSA cyclization through a standard spectrophotometer. Since manual mixing of the reaction requires several seconds, I carried out also fast kinetic measurements with a stopped-flow. Even stopped-flow reaction showing no lag phase in a mOAT coupled assay. In this condition the turnover number of mOAT is about 3.3 s^{-1}, that is the first turnover of the enzyme is expected to occur

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in 0.3 s and as no lag was observed it is possible deduce that cyclization occurs spontaneously at a rate substantially greater than 3 s^{-1}.

Phosphonates are very abundant and important compounds thanks to their highly stable covalent C-P bond. Their role in nature is still poorly understood but to date we know they are used as protective structural elements, are important secondary metabolites and, for many marine microorganisms, they also represent a powerful alternative phosphorus source. Furthermore, in recent years they have acquired a great industrial relevance with application ranging from agrochemistry to medical (glyphosate, fosfomycin, etc.). While the phosphonates biosynthesis pathway is initially the same, the biodegradation involves several very specialized strategies and very unusual enzymatic pathways to cleave the P-C bond. Thus, understanding the mechanism of known and newly discovered pathways will help to solve the complete metabolic cycles of phosphonates and hence make way for new applications and contain their possible negative effects on the environment. For this purpose, I analyzed the genetic contexts of known enzymes belonging to CP-hydrolase pathway, characteristic of the aminophosphonates degradation, which are compounds with primary amine group (as amino acids), and therefore potential substrates of PLP-dependent enzymes. Through this analysis I identified a gene sequence, coding a hypothetical PLP-dependent GABA transaminase (GABA-T), whose orthologs are present in two distinct operons dedicated to the degradation of ciliatine (the most abundant aminophosphonate in nature), phnWX and phnWAY, spread respectively in alpha and gamma proteobacteria. Since phnW is a PLP-dependent transaminase that catalyzes the transfer of the amino group of ciliatine to pyruvate to give phosphonoacetaldehyde and alanine, it is unlikely that also the hypothetical GABA-T performs the same reaction. It is more likely that the enzyme can form ciliatine or phosphonoacetaldehyde from other aminophosphonate, which makes it even more difficult to characterize (due to a deficiency of many synthetic phosphonic acids and of their enantiomerically pure form). To characterize the activity of this enzyme, all the proteins of the *Vibrio splendidus* cluster genes (phnW, Hyp GABA-T and phnX) were synthesized in the appropriate bacterial expression vectors (pET system), then they were overexpressed in different BL21(DE3)-derived strains and purified trough affinity chromatography. I set up a coupled assay with phnW, phnX and alcohol dehydrogenase (ADH) that allows to monitor the formation of phosphonoacetaldehyde, produced from ciliatine, converted to acetaldehyde by phnX which in turn is an ADH substrate. Preliminary analysis reveal that Hyp GABA-T has a weak transaminase activity against phosphonoalanine and some amino acids (L-alanine, L-glutammate, L-aspartate), not against ciliatine. Its transaminase activity towards phosphonoalanine doesn't explain its presence in this operon. To investigate if Hyp GABA-T can react on other potential substrates (aminophosphonate) and/or through other mechanism of action I will add it to the coupled assay.

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