INVITED REVIEW

Phosphorylation of pyridoxal 5′-phosphate enzymes: an intriguing and neglected topic

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Abstract

Pyridoxal 5′-phosphate (PLP)-dependent enzymes catalyze a wide range of reactions of amino acids and amines, with the exception of glycogen phosphorylase which exhibits peculiar both substrate preference and chemical mechanism. They represent about 4% of the gene products in eukaryotic cells. Although structure–function investigations regarding these enzymes are copious, their regulation by post-translational modifications is largely unknown. Protein phosphorylation is the most common post-translational modification fundamental in mediating diverse cellular functions. This review aims at summarizing the current knowledge on regulation of PLP enzymes by phosphorylation. Starting from the paradigmatic PLP-dependent glycogen phosphorylase, the first phosphoprotein discovered, we collect data in literature regarding functional phosphorylation events of eleven PLP enzymes belonging to different fold types and discuss the impact of the modification in affecting their activity and localization as well as the implications on the pathogenesis of diseases in which many of these enzymes are involved. The pivotal question is to correlate the structural consequences of phosphorylation among PLP enzymes of different folds with the functional modifications exerted in terms of activity or conformational changes or others. Although the literature shows that the phosphorylation of PLP enzymes plays important roles in mediating diverse cellular functions, our recapitulation of clue findings in the field makes clear that there is still much to be learnt. Besides mass spectrometry-based proteomic analyses, further biochemical and structural studies on purified native proteins are imperative to fully understand and predict how phosphorylation regulates PLP enzymes and to find the relationship between addition of a phosphate moiety and physiological response.

Abbreviations

PLP	Pyridoxal 5′-phosphate
PTM	Post-translational modification
TAT	Tyrosine aminotransferase
GABA	γ-Aminobutyric acid
GABA-T	γ-Aminobutyric acid transaminase
SPT	Serine palmitoyl transferase
GAD	Glutamate decarboxylase
DDC	DOPA decarboxylase
AADC	Aromatic amino acid decarboxylase
HDC	Histidine decarboxylase
CSAD	Cysteine sulfinic acid decarboxylase
SR	Serine racemase
CBS	Cystathionine β-synthase
ODC	Ornithine decarboxylase
PD	Parkinson's disease
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
CaMKII	Calcium–calmodulin dependent protein kinase II
LBC1	Long-chain base 1

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LBC2   Long-chain base 2
HSAN1   Autonomic neuropathy type 1
NMDA   *N*-methyl-D-aspartate

Introduction

One of the main reasons for the extreme flexibility of proteins is the ability to respond to different cell environmental conditions, being modified and regulated by many factors. The regulation of enzyme activity in vivo by post-translational modification (PTM) provides a rapid way for cells to respond to changing physiological conditions, by modulating physico-chemical features, conformation, stability and activity, thus leading to a global altered protein function. PTMs are well-known mechanisms that trigger subtle changes in proteins and make them more suitable to face particular conditions.

Modern proteomic high-throughput mass spectrometry methods have permitted the identification of more than 200 different types of PTMs (Mann and Jensen 2003; Lu et al. 2013) spanning from phosphorylation, glycosylation, fatty acid linkage for membrane attachment, methylation, nitrosylation, glutathionylation, acetylation, ubiquitinylation, and many others (Seo and Lee 2004). Many platforms and databases have been developed to map all experimentally found modification consensus sequences to allow prediction of the sites more prone to specific PTM in every protein.

Phosphorylation was one of the first PTMs to be identified and is one of the most widespread, versatile and studied PTMs (Cohen 2000, 2002). It is a reversible covalent modification that could alter the function, the binding partners and the localization of proteins, thus determining the fine tuning of their biological activity. It is implied in many cellular processes such as signal transduction pathways, growth, differentiation and apoptosis. It has become more and more evident that alterations in phosphorylation pathways could cause or worsen pathological conditions such as cancer (Singh et al. 2017). This modification is carried out by a balanced interplay between kinases that phosphorylate a substrate protein using ATP as co-substrate, and phosphatases that are responsible for the removal of the phosphate (Hunter 1995). Phosphorylation commonly occurs on the hydroxy group of serine, threonine or tyrosine residues. There are 518 protein kinases known in the human genome, of which 428 are known or predicted to phosphorylate serine or threonine, while the other 90 react with tyrosine (Manning et al. 2002). The phosphorylation event could take place at only one or at multiple sites on a specific protein. Furthermore, a protein could be a substrate of a single or of multiple kinases, thus establishing complex cascade networks in response to a stimulus.

Because of their importance, kinases have been the subject of numerous studies on their classification and mechanism of action (Kornev et al. 2006; Taylor and Kornev 2011). It has been recently reported that many eukaryotic proteins (probably thousands) undergo phosphorylation during their lifespan (Venerando et al. 2017). Among them, pyridoxal 5′-phosphate (PLP)-dependent enzymes should not represent an exception. However, data concerning phosphorylation of PLP-enzymes are scarce: a search of the literature reveals that only 11 PLP-enzymes are known to be subject to phosphorylation.

From a structural point of view, PLP-enzymes are classified into five different fold types (from I to V) depending on their amino acid sequence, secondary structure and known spatial structures (Grishin et al. 1995; Schneider et al. 2000). Fold I, the largest, is the α-family, also known as the aminotransferase superfamily. Fold II is also known as the β-family, since many of its members catalyze reactions at the β-carbon. Fold III is the alanine racemase family, primarily containing racemases and some decarboxylases. Fold IV contains only D-amino acid and branched-chain amino acid aminotransferases. Glycogen phosphorylase is the only member of Fold V. The organization in fold types is useful to understand some structural constraints of the active site, the identity of the residues involved in catalysis and the conformational changes carried out by these enzymes upon cofactor and/or substrate binding. Despite their great numbers, wide distribution (Percudani and Peracchi 2003) and relevance for amino acid metabolism, only a few PLP-dependent enzymes have been shown to undergo phosphorylation. The aim of this review is to outline what is known at present about the involvement and the role (if already determined) of phosphorylation in the regulation of PLP enzymes that are subjected to it.
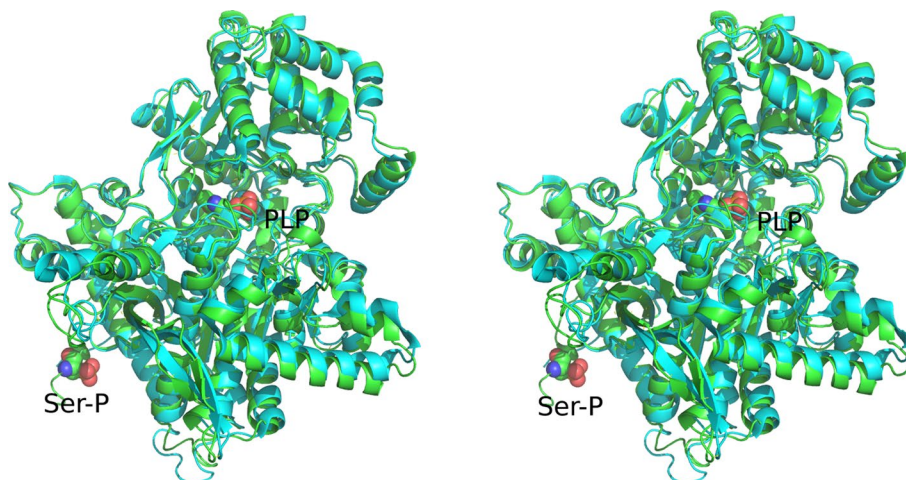
Glycogen phosphorylase

One of the best studied and classic examples of protein phosphorylation deals with a PLP-dependent enzyme, namely glycogen phosphorylase. The pioneering work carried out from the late 1930s to late 1950s by Carl and Gerty Cori, by Burnett and Kennedy, and finally by Fischer and Krebs, led to the demonstration that muscle glycogen phosphorylase exists in two forms (*a* and *b*), with the less active *b* form being converted into the active *a* form by phosphorylation, as well as by AMP binding (Johnson 1992). Glycogen phosphorylase is a unique PLP enzyme, since the substrate, glycogen, is a polysaccharide, and the phosphorolysis reaction is carried out by the 5′-phosphate group of the cofactor that has been proposed to behave in this case as an acid–base catalyst (Palm et al. 1990). However, a study with PLP phosphate analogs with altered pK_as suggested that the phosphate

remains a dianion throughout catalysis, and therefore, provides electrostatic rather than acid–base catalysis (Stirtan and Withers 1996). This is a property not shared by all the other PLP-dependent enzymes, which act on amino acids and amines, and utilize the aldimine and the conjugated pyridine ring as electron sinks, thus promoting catalysis. In these latter enzymes, the phosphate group functions mainly as a handle for the enzyme to bind the cofactor during the catalytic cycle, although in some cases it may also participate in catalysis (Phillips et al. 2014).

Glycogen phosphorylase exhibits three tissue-specific isozymes, found in muscle, liver and brain. Although the three isozymes have high sequence homology, they show significant differences in regulation. Liver glycogen phosphorylase is primarily regulated by phosphorylation on Ser-14 by phosphorylase kinase, in response to glucagon. Due to its key role in releasing glucose from the liver, this enzyme has emerged recently as a potential target for Type 2 antidiabetic drugs. Muscle glycogen phosphorylase is activated by both phosphorylation as well as ligands such as AMP, whereas brain glycogen phosphorylase is not activated by phosphorylation, but is activated by ligands such as AMP (Mathieu et al. 2017). The crystal structures of muscle and liver glycogen phosphorylase *a* and *b* have been determined (Sprang et al. 1988). The protein is a dimer and is allosterically activated by phosphorylation. In muscle glycogen phosphorylase, phosphorylation of Ser-14 results in conformational changes near the active site, despite a distance of about 40 Å between the serine phosphate and PLP (Fig. 1). In fact, in phosphorylase *b*, Ser-14 is in a disordered region and is not observed in the structure. The AMP-binding site is located near the phosphorylation site, and AMP binding results in similar structural changes as phosphorylation. These structural changes resulting from phosphorylation of Ser-14 of glycogen phosphorylase provide a paradigm for the structural effects of phosphorylation of other PLP-dependent enzymes.

PLP-dependent reactions of amino acids

The great majority of PLP enzymes act on amino acids rather than sugars with a general mechanism depicted in Fig. 2. The cofactor is covalently bound to the protein moiety through a Schiff base linkage of its aldehyde group with the ε-amino group of a lysine, forming the so-called internal aldimine. When an amino acid enters the active site, its α-amino group substitutes the ε-amino group of the lysine residue in a transaldimination reaction that leads to the *gem*-diamine that is subsequently converted into an external aldimine. From this step on, the reaction pathway to be undertaken depends on which of the bonds of the external aldimine is perpendicular to the imine-pyridine plane [Dunathan hypothesis (Dunathan 1966)]. This explains the enormous variety of reactions that could be catalyzed by PLP enzymes (Phillips 2015): transamination, α- and β-decarboxylation, β- and γ-elimination, racemization, β- and γ-substitution, retro-aldol cleavage and even oxidation.

In addition to the well-known glycogen phosphorylase, also tyrosine aminotransferase (TAT), γ-aminobutyric acid (GABA) aminotransferase (GABA-T), serine palmitoyltransferase (SPT), glutamate decarboxylase (GAD), DOPA decarboxylase (DDC), histidine decarboxylase (HDC), cysteine sulfinic acid decarboxylase (CSAD) belonging to fold-type I, serine racemase (SR) and cystathionine β-synthase (CBS) to fold-type II, and eukaryotic ornithine decarboxylase (ODC) to fold-type III (Grishin et al. 1995), have been found to be phosphorylated.
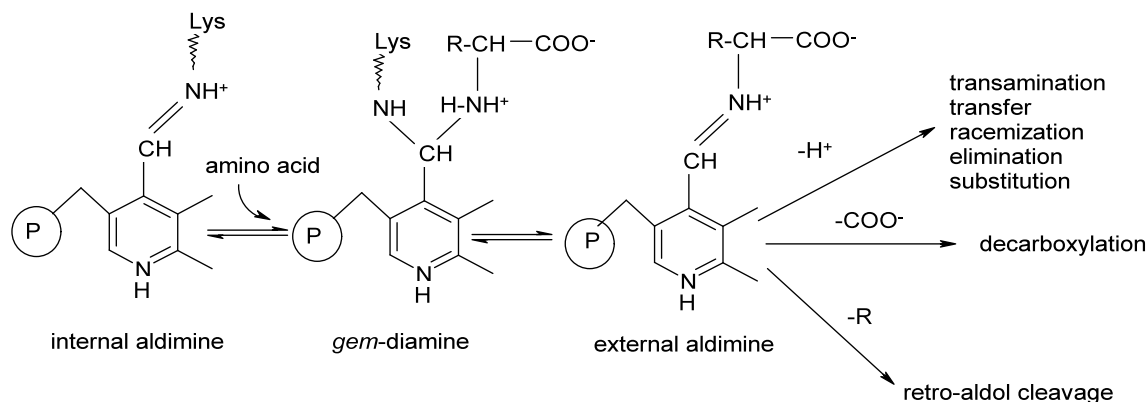
Fig. 1 Structure of Ser-14 phosphorylated glycogen phosphorylase. The structure of one subunit of rabbit muscle glycogen phosphorylase *a* overlaid on glycogen phosphorylase *b*, showing the position of the phosphoserine-14 and PLP as space-filling models. The green ribbon is phosphorylase *a* and the cyan ribbon is phosphorylase *b*. The figure was prepared with Pymol (the PyMOL Molecular Graphics System, version 1.7.2.1 Schrödinger, LLC) from protein structure files 1GPA and 1GPB (color figure online)

Fig. 2 Mechanism of reactions of PLP-enzymes. The cofactor PLP is covalently linked to the ε-aminic group of a lysine residue in the active site of a resting PLP enzyme in the so-called internal aldimine species. When an amino acidic substrate enters the active site, it makes a nucleophilic attack to the 4′-carbon of PLP generating a sp^3 species: the *gem*-diamine, which is subsequently converted into the external aldimine by substituting the ε-amino group of the lysine with the α-amino group of the substrate. Then, the reaction is directed into a precise direction depending on which of the bond of the α-carbon is perpendicular to the imine-pyridine plane and is thus chemically prone to be broken. This leads to the high versatility of reactions catalyzed by PLP enzymes. This mechanism is drawn according to the so-called Dunathan hypothesis (Dunathan 1966) and a huge amount of information on PLP enzymes recently revised by (John 1995; Phillips 2015)

Fold-type I decarboxylases: GAD, DDC, HDC, and CSAD

A recent review on this group of enzymes reveals many similarities as well as their major differences and specific features (Paiardini et al. 2017). Since these decarboxylases are responsible for the synthesis of essential molecules (neurotransmitters, bioactive amino acids and polyamines), it is conceivable that their activity should be finely tuned to respond to physiological conditions. Here, we review evidence that these enzymes can be phosphorylated, suggesting a possible interconnected regulation mechanism.

Among these decarboxylases, the most well-known is GAD, which catalyzes the a-decarboxylation of L-glutamate to GABA and is found in both prokaryotes and eukaryotes. In most vertebrates, the enzyme exists on two functional dimeric isoforms (GAD65 and GAD67) (Fenalti et al. 2007), while in plants (Gut et al. 2009; Astegno et al. 2015, 2016) and in *E. coli* and other enteric bacteria, it is characterized by a hexameric assembly (Capitani et al. 2003).

In mammals, GABA is a key inhibitory neurotransmitter in central nervous system and plays many fundamental roles in motor control, vision, as well as in brain plasticity-related processes such as memory, learning, locomotion, and during the development of the nervous system (Roberts 1975; Tower 1976; Hornykiewicz et al. 1976). It is also widely recognized that many clinical conditions including psychiatric disorders, spasticity, epilepsy, stiff-person syndrome, anxiety and cerebral ischemia involve imbalances in excitation and inhibition where GABA production is fundamental (Blum and Jankovic 1991; Sherman et al. 1991; Arias et al. 1992; Soghomonian and Laprade 1997; Kash et al. 1997).

The two human GAD isoforms, the membrane-anchored GAD65, and the cytosolic GAD67 (Erlander et al. 1991; Bu et al. 1992), are products of two independently regulated genes and expressed in different cerebral regions. Both of them utilize PLP as cofactor to perform catalysis. However, each of the two isoforms presents particular characteristics. GAD67 is present as holoenzyme, with the PLP cofactor bound as internal aldimine, while GAD65 is instead 50% in the apo form without bound PLP (Martin and Rimvall 1993). This was interpreted as suggesting GAD67 is responsible for basal GABA production, while GAD65 can be rapidly activated when higher neurotransmitter levels are required and cycles between an active holo form and an inactive apo form (Fenalti et al. 2007). Despite the high sequence identity and structural similarity of the two isoforms (Fenalti et al. 2007). GAD65 is an antigen in patients with type 1 diabetes and other autoimmune disorders, while GAD67 is rarely autoantigenic (Baekkeskov et al. 1987, 1990; Paiardini et al. 2017; Jayakrishnan et al. 2011). Recently, through a combined computational and experimental approach, it has been advanced that production of GAD65 autoantibodies could be related to cofactor-controlled conformational changes, due to the flexibility of some GAD65 structural elements, such as the C-terminal domain and the catalytic loop with respect to GAD67 (Kass et al. 2014; Langendorf et al. 2013). These determinants have been proposed to represent molecular bases for regulation of GABA production (Fenalti et al. 2007; Langendorf et al. 2013).

Both GAD65 and GAD67 have been reported to undergo phosphorylation (Bao et al. 1995; Hsu et al. 1999). Surprisingly, this modification exerts opposite functional effects for the two isoforms, since GAD65 is activated by phosphorylation while GAD67 is inhibited (Wei et al. 2004). The treatment in vitro of both GAD isoforms with three protein kinases (PKA, PKC and CaMKII) showed that PKCε is responsible for GAD65 phosphorylation, while PKA phosphorylates GAD67, and that in GAD67, Thr-91 is subjected to this modification (Wei et al. 2004). Similar to glycogen phosphorylase, the phosphorylation site in GAD67 is > 40 Å from the PLP (Fig. 3). Unfortunately, the structure is truncated on the N terminus, and Thr-91 is not visible in the structure. A model was proposed, taking into account the different regulation to which the two GAD proteins are subjected, due probably to their different responses to physiological conditions (Wei et al. 2004; Jin et al. 2003). In particular, it has been suggested that phosphorylation could alter the K_m for glutamate in a different manner for the two isoforms. In addition, the neuronal stimulation on the one hand activates both PKA and PKCε which phosphorylate GAD67 and GAD65, respectively, leading to an increase in GABA levels in vesicles which could then be released (Wei et al. 2004; Jin et al. 2003). However, it should be taken into account that the kinases and phosphatases that are involved in vivo are unknown and their identification represents an essential step for dissecting the signal pathways that regulate GABA neurotransmission (Wei and Wu 2008). A number of putative phosphorylation sites for both GAD65 and GAD67 have been proposed by means of bioinformatic prediction (Wei and Wu 2008). The effects of phosphorylation are not only limited to altered enzymatic activity but also to compartment localization. Phosphorylation of serine residues 3, 6, 10 and 13 in GAD65 regulates membrane anchoring without exerting any effect in catalysis (Namchuk et al. 1997). This has been proposed to be related to higher GAD65 membrane association under neuronal stimulation (Wei and Wu 2008). Other phosphorylation sites on GAD65 have been

recently identified, unraveling the importance of Thr-95 in regulating activity and in the interplay with GAD67 following neuronal stimulation (Chou et al. 2017).

DDC (also known as aromatic amino acid decarboxylase, AADC) is a structurally similar α-decarboxylase whose role is the synthesis of the neurotransmitters, dopamine and serotonin, from the corresponding amino acids, L-Dopa and 5-hydroxytryptophan. DDC activity is reduced in regions of the brain in Parkinson's disease (PD), thus leading to low levels of dopamine. Pharmaceutical treatment of PD in the early stages is with L-Dopa and a peripheral DDC inhibitor such as carbidopa or benserazide. Information regarding phosphorylation of DDC is relatively scarce. A few papers have been published reporting that both recombinant and immunoprecipitated (from brain homogenates) DDC could be phosphorylated by the catalytic subunits of cyclic AMP-dependent protein kinase, and that the enzymatic activity increases in both cases (70% for the recombinant and 20% for the immunoprecipitated enzyme) (Duchemin et al. 2000). Moreover, interaction with α-synuclein, implicated in PD, has been reported to reduce phosphorylation levels of DDC (by 1.5-fold) probably by activation of a phosphatase such as protein phosphatase 2A (Tehranian et al. 2006). Concomitantly, a-synuclein leads to inhibition of DDC activity, a possible mechanism of dopamine homeostasis which is compromised in PD pathogenesis (Tehranian et al. 2006). Finally, cyclic guanosine monophosphate/protein kinase G (cGMP/PKG) has also been determined to phosphorylate DDC, increasing V_{max} by ~ 30% and K_m by ~ 60% (Duchemin et al. 2010). Activation of neuronal DDC by drugs that increase phosphorylation or allosteric activators could be a novel approach to treatment of PD. It is conceivable that an alteration or, more specifically, a decrease in phosphorylation level of DDC could be also involved in dysregulation of dopamine and serotonin observed in AADC deficiency, a rare genetic disease affecting *DDC/AADC* gene, causing neurological damages. In this sense, a treatment agent that acts increasing phosphorylation level could also be

Fig. 3 Structure of Thr-91-phosphorylated glutamate decarboxylase. The structure of the dimer of GAD67, showing the relationship between the phosphorylation site and the active site. The N terminus of the protein was truncated at Thr-93, so the phosphorylation site at Thr-91 is not seen. The figure was prepared with Pymol (the PyMOL Molecular Graphics System, version 1.7.2.1 Schrödinger, LLC) from protein structure file 2OKJ
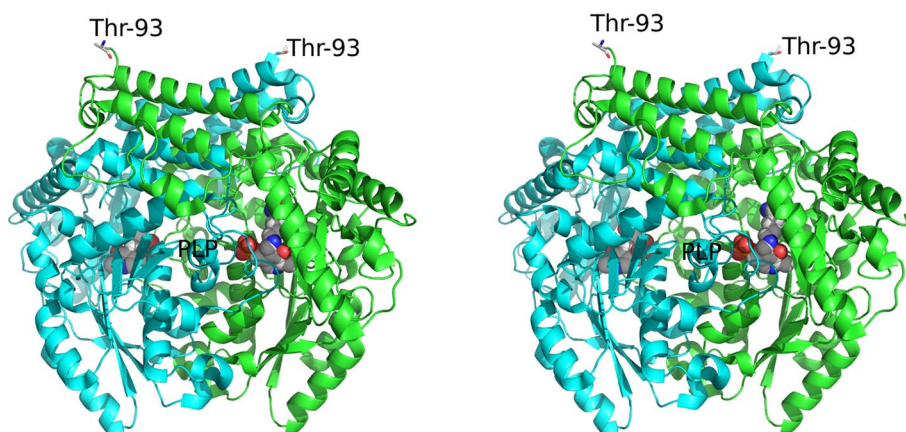
beneficial for patients carrying this genetic disease. However, no phosphorylation sites of DDC have been identified until now.

HDC is the only component of the biosynthetic pathway of histamine, an important biogenic amine with physiological regulatory roles in neurotransmission, gastric acid secretion and immune response. Thus, impairment of histamine metabolism is related to many pathological states such as inflammatory responses, peptic ulcer and several central nervous system disorders (Ohtsu 2010; Panula and Nuutinen 2013). More recently, new interesting relationships have been established between HDC expression and growth of different carcinoma types and neuroendocrine tumours, especially gastrointestinal cancers (Kennedy et al. 2012), especially cholangiocarcinoma (Francis et al. 2012). HDC is expressed as a 74 kDa inactive polypeptide (Fleming et al. 2004) and only after post-translational proteolysis of the C-terminal part, probably by caspase-9, the enzyme results in 53–55 kDa active isoforms (Dartsch et al. 1998). Since 30 years ago, papers have been published regarding possible HDC regulation by phosphorylation in rat hypothalamus or gastric mucosa extracts, with conflicting results. In vitro, hypothalamic HDC is inhibited by incubating the homogenate under phosphorylating conditions (ATP, cAMP, and Mg^{2+}) in the presence of a cAMP-dependent protein kinase (Huszti and Magyar 1984), and this effect is reversed by the addition of cAMP-dependent protein kinase inhibitor, enhancing HDC activity above control levels (Huszti and Magyar 1985). Although similar results are found also with the partially purified hypothalamic enzyme (Huszti and Magyar 1987), the incubation of gastric supernatant with various combinations of ATP, Mg^{2+}, cAMP and protein kinase under the blockade of endogenous phosphatases fails to alter significantly the enzyme activity. On the other hand, fractionated rat gastric mucosa enzyme distributes into multiple forms with different charges (Savany and Cronenberger 1982) and with the generation of less-negatively charged species in a time and temperature-dependent manner (Savany and Cronenberger 1988). In addition, in absence of PLP, gastric HDC is reversibly inactivated by phosphatase in a time and dose-dependent process (Savany and Cronenberger 1989) leading to the suggestion that dephosphorylation promotes and/or stabilizes apoenzyme formation. In fact, full reactivation is achieved by the addition of PLP, that at the same time reduces the number of forms with low negative charge (Savany and Cronenberger 1990), suggesting a relationship between the charge heterogeneity and the phosphorylation state.

CSAD is a recently identified decarboxylase involved in the production of taurine, which is involved in many biological processes. It has been reported that CSAD is activated when phosphorylated and inhibited when dephosphorylated. PKC is responsible for the phosphorylation reaction, while

protein phosphatase 2C for phosphate removal (Tang et al. 1997). In particular, a neuronal stimulus resulting in taurine release, increases also taurine synthesis by activating PKC which in turns phosphorylates CSAD leading to an increase of taurine levels (Tang et al. 1997; Wu et al. 1998). This behaviour reminiscent of that of GAD65 and opposite to that of GAD67, highlighting the interplay of taurine and GABA in brain.

Fold-type I aminotransferases and acyltransferases: TAT, GABA-T and SPT

Phosphorylation of TAT and GABA-T was reported in papers published more than 40 years ago, in particular TAT is found phosphorylated in vivo and it is demonstrated that this modification does not alter enzyme activity (Lee and Nickol 1974). TAT is an enzyme active in the liver and whose role is to regulate the catabolism of tyrosine and phenylalanine. Its expression is modulated by cAMP and glucocorticoids in primary hepatocytes (Schmid et al. 1987) and cAMP-dependent protein kinase is considered to be responsible for phosphorylation (Spielholz et al. 1984; Pogson et al. 1986). The same kinase acts also on purified pig brain GABA-T and no effect of phosphorylation on enzyme kinetic properties is reported (Carr et al. 1986), even if no in-depth investigations on these phosphorylated aminotransferases have been carried out since then. Although no effects of phosphorylation on activity are seen with these enzymes in vitro, it is possible that phosphorylation can affect the interaction with other cell components in vivo, or their intracellular lifetime.

Some papers have recently been published on the phosphorylation of SPT, the first and rate-limiting enzyme of de novo sphingolipid biosynthetic pathway, that catalyses the condensation between L-serine and an acyl-CoA thioester substrate (typically palmitoyl-CoA). Despite the identification of some associated regulatory subunits, its core is a heterodimer composed by the subunits long-chain base 1 and 2 (LCB1 and LCB2) encoded by separate paralogous genes (Yard et al. 2007). Although only LCB2 contains the PLP-dependent catalytic site, LCB1 plays important roles in the regulation of the enzyme. Point mutations in both subunits are, indeed, associated with hereditary sensory and autonomic neuropathy type 1 (HSAN1), an autosomal-dominant genetic disorder characterized by peripheral neuropathy with signs of neuronal degeneration and minor limb injury development into extensive ulcerations with necessary amputation. HSAN1 is mainly due to a SPT substrate shift from L-serine to L-alanine, with consequent decrease in sphingolipids, and therefore, myelin production and simultaneously accumulation of atypical neurotoxic 1-deoxy-sphingolipids (1-deoxySL) (Penno et al. 2010). In

addition, wild-type SPT can metabolize L-alanine under certain conditions, associated with metabolic syndrome, type 2 diabetes and diabetic neuropathy (Othman et al. 2012, 2015). In particular, a fraction containing wild-type SPT extracted from CHO cells is found to be phosphorylated at Ser-384 of LCB2 subunit and phosphorylation is lost after alkaline phosphatase treatment. In addition, the mutation of this residue to phenylalanine, with the loss of the phosphorylation site, switches the substrate specificity of the enzyme from L-serine to L-alanine (Ernst et al. 2015). Despite the evidence of the alternative substrate preference of SPT, the physiological role of this dynamic regulation is still unknown. SPT phosphorylation was also found in BCR-ABL positive cells, a condition linked to the development of chronic myeloid leukemia (Taouji et al. 2013). LCB1, the other SPT subunit, is indeed phosphorylated at Tyr-164 in this cell types under basal condition and the inhibition of BCR-ABL with Imatinib decreases SPT phosphorylation, leading to a time and dose-dependent activation of the enzyme and its translocation from the endoplasmic reticulum to the Golgi apparatus. In addition, Tyr-164 mutation induces apoptosis in BCR-ABL-positive leukemia cells, identifying SPT as a potential therapeutic target to overcome Imatinib resistance.

Fold-type II: SR and CBS

L-Serine is racemized by SR to give D-serine, which binds to the glycine site as a co-agonist of the *N*-methyl D-aspartate (NMDA) receptors in various regions of the brain, exerting its effects in neurotoxicity and synaptic plasticity. Given the involvement of D-serine in neurodegenerative diseases and schizophrenia, the regulation by phosphorylation may be relevant in physiological and pathological mechanisms. SR is a tightly regulated enzyme by various factors: nucleotides, divalent cations, *S*-nitrosylation, other protein partners, and phosphorylation (Baumgart and Rodriguez-Crespo 2008). It was shown that phosphorylation at Thr-277 is typical of the 10% pool of membrane-associated mouse SR in non-neuronal cells (Balan et al. 2009). Mouse cytosolic SR has been found to be phosphorylated at Thr-71 and the enzymic activity, in terms of V_{max}, is increased following phosphorylation (Foltyn et al. 2010). This represents the main phosphorylation site on SR. Moreover, it has been demonstrated that PKC controls rat SR phosphorylation and thus D-serine production both in vitro and in vivo (Vargas-Lopes et al. 2011). Interestingly, this phosphorylation event inhibits SR in astrocytes and neurons (Vargas-Lopes et al. 2011). Since neither Thr-71 nor Thr-227 are conserved in human SR (the human corresponding residues are Ala-71 and Met-227), a search in phosphoproteomic analyses reveals that the human enzyme possesses multiple possible phosphorylation sites conserved also in mouse and rat: Ser-134, Tyr-207,

Ser-212, Tyr-218, Ser-220, Ser-242 and Ser-339 (Klammer et al. 2012; Mertins et al. 2016). No in-depth investigation exists yet on the role of these residues regarding the structure–function relationship of SR.

Phosphorylation of human CBS has been reported in very recent papers (d'Emmanuele di Villa Bianca et al. 2015, 2016). Human CBS is a unique heme-containing enzyme that catalyzes the PLP-dependent condensation of homocysteine with serine to form cystathionine (Miles and Kraus 2004). Cystathionine is then cleaved by cystathionine γ-lyase (CGL) to give cysteine. CBS represents the first step in the transsulfuration pathway which connects the methionine cycle to cysteine production, therefore, its proper function is crucial for both cysteine and methionine metabolism. Accordingly, a compromised CBS activity or the absence of CBS leads to manifestation of CBS-deficient homocystinuria, a condition characterized by very high levels of plasma total homocysteine and methionine.

Recent studies have reported that the activity of human CBS within the urothelium, the epithelial lining the inner surface of human bladder, is regulated by phosphorylation (d'Emmanuele di Villa Bianca et al. 2015). The authors have demonstrated that CBS activity is specifically enhanced by phosphorylation at Ser227 in cGMP/PKG-dependent mechanism (d'Emmanuele di Villa Bianca et al. 2015, 2016). CBS is markedly expressed in the human urothelium (d'Emmanuele di Villa Bianca et al. 2015) representing the main enzymatic source of H_2S, which has been recently proposed as a new signal molecule in the control of bladder tone. In this context, the finding that CBS phosphorylation by PKG increases endogenous H_2S production (d'Emmanuele di Villa Bianca et al. 2015) suggests the possibility that the regulation of H_2S formation by CBS may involve CBS phosphorylation. Interestingly, CBS was found to be phosphorylated also on Ser-525 in a PKG-dependent manner, but without affecting H_2S production. This could be due to the localization of Ser-525 in the C-terminal regulatory domain which is the binding region of the allosteric activator *S*-adenosyl methionine. Therefore, phosphorylation of this serine residue may be implicated in the modulation of further features of CBS protein. Additional experiments are necessary to unravel the molecular mechanism underlying CBS activation by phosphorylation.

Fold-type III decarboxylases: eukaryotic ODC

ODC is the rate-limiting enzyme in polyamine biosynthesis, which is essential for cell division. The mammalian enzyme presents a consensus phosphorylation sequence for protein casein kinase II, and it has been reported that ODC, either recombinant mouse enzyme, or from intact mouse

overproducing ODC cells, can be phosphorylated at Ser-303 by casein kinase II (Rosenberg-Hasson et al. 1991). Studies with the wild-type and the S303A variant show that phosphorylation does not affect ODC activity as well as protein rate turnover (Rosenberg-Hasson et al. 1991). Reddy et al. (1996) reported that ODC could undergo multisite phosphorylation (in addition to that performed by casein kinase II) involving unidentified kinases that leads to ~ 50% increase in the V_{max} value and higher protein stability. Again, phosphorylation may affect the enzyme stability in vivo despite no direct effect on activity.

Concluding remarks and future perspectives

A search of the human phosphoproteomic data reveals that, although theoretically many PLP enzymes appear to be involved in phosphorylation regulatory pathways, only few of them are reported to be phosphorylated, at least until now. Apart from glycogen phosphorylase, for many of the others, the knowledge of how this modification affects enzyme function is unknown. Here, we show how difficult it is to interpret the possible modulation exerted by this modification, even for those enzymes whose structure is solved and the phosphorylation site(s) identified, as for example GAD or SR. The common feature appears to be the exposed position of the phosphorylation site in both enzymes. Moreover, the same modification could trigger different effects. This is also evident for these investigated PLP-enzymes where phosphorylation can activate, inhibit or have no effects. The relation among phosphorylation site, structural modification and functional effect is desirable to be obtained to shed light into complex metabolic regulation networks in which these enzymes are involved and often responsible for the first committed step in biosynthetic reactions of essential bioactive amines.

Since phosphorylation is a common and widespread strategy of regulation and interplay among transduction and metabolic pathways, it is of much interest to deepen the knowledge for PLP enzymes that represent the 4% of the enzyme activities present in a eukaryotic cells (Percudani and Peracchi 2003, 2009).

In perspective, the unraveling of the structure-to-function relationships of phosphorylation events for all known PLP enzymes and especially for those placed in key knots of physiological controlling routes is highly attractive. In addition, this information could be of great help also for PLP enzymes involved in hereditary pathogenic diseases, to concur in understanding complex phenotypes of patients bearing mutations that apparently lead to subtle modifications in protein structures. The study of regulation of PLP enzymes is at its beginning and needs to be further systematically undertaken.

Compliance with ethical standards

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