Pyroglutamic acid: throwing light on a lightly studied metabolite

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Pyroglutamic acid or 5-oxoproline is the cyclic lactam of glutamic acid. Its presence in living cells has been reported from archaebacteria to humans, and its occurrence in living cells has been known for over a century. Despite its almost ubiquitous presence, the role of pyroglutamic acid in living cells is poorly understood. Pyroglutamic acid is found as an N-terminal modification in many neuronal peptides and hormones that also include the accumulating peptides in Alzheimer's disease and familial dementia. The modification is also observed in proteins that include many antibodies, some enzymes and structural proteins. The modification in proteins has been shown to contribute to both the structural and activity-related properties of the proteins. Pyroglutamate also exists as a free metabolite in living cells. In several genetic disorders of humans, and in an acetaminophen-induced metabolic disorder, high levels of pyroglutamic acid are secreted in the urine in what is known as 5-oxoprolinuria. The proposed functions of free pyroglutamic acid include its role as an analogue or reservoir of glutamate, as well as other functions unique to it, that includes a possible role in osmoprotection. This short review tries to capture our current understanding of pyroglutamic acid in living cells.

Keywords: γ-Glutamyl cycle, neuronal peptides, pyroglutamic acid, 5-oxoproline, 5-oxoprolinuria.

Introduction

PYROGLUTAMIC acid is also known as 5-oxoproline pyrrolidone 2-carboxylic acid, and is the cyclic lactam of glutamic acid (Figure 1). Pyroglutamic acid was first discovered by Haitinger[1] in 1882, who found that when heated at 180°C, glutamate is converted into pyroglutamate by losing a molecule of water. For a long time pyroglutamic acid formation in tissues was attributed to non-enzymatic, spontaneous formation from glutamine and glutamate, and it was not until over 5 decades later that the enzymatic formation of this metabolite from glutathione (by an enzyme that is now γ-glutamyl cyclotransferase) in living tissues was demonstrated[2,3]. Subsequently, a pyroglutamic acid-cleaving enzyme was discovered and given the name 5-oxoprolinase[4]. Alton Meister[5], who strode like a giant across this field, integrated this synthesis and degradation of pyroglutamic acid into the cycle of synthesis and degradation of glutathione, the γ-glutamyl cycle in the 1970s. Following the description of the cycle, however, biochemical interest in 5-oxoproline, the enzymes and the metabolic interactions has been subdued.

The call by Moret and Briley[6], many years ago, for more research on pyroglutamic acid was only met by an admonishing comment by Christensen[7] that it should not be referred to as an amino acid, owing to its non-zwitterionic nature, although not commenting on the main purpose of the Moret and Briley article[6]. Garattini[8], in a keynote presentation at an international symposium on glutamate also addressed the need for greater research on pyroglutamatic acid, but again these remarks appear to have been largely unheeded.

The paucity of biochemical research on pyroglutamic acid is reflected in the fact that γ-glutamyl cyclotransferase, the enzyme principally thought to form 5-oxoproline, was biochemically purified in 1969 by Orlowski *et al.*[9], but the identity of the protein became known[10] only in 2008. Similarly, although the 5-oxoprolinase enzyme had been purified four decades ago[4], and the gene identified[11] in 1996, recombinant expression of a eukaryotic 5-oxoprolinase was only achieved in 2010, along with the first structure–function studies of this interesting enzyme[12].

One reason for this might be the general view that pyroglutamic acid is largely an intermediate metabolite. A second reason for the retarded research in this area has been the absence, till recently, of a robust and sensitive enzyme assay for pyroglutamic acid and 5-oxoprolinase.

We describe here the current status of pyroglutamic acid research, the new developments and insights, and why more research is needed in this area.

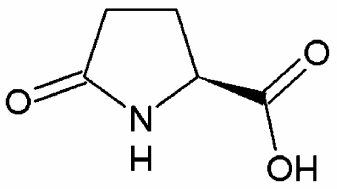

Figure 1. Chemical structure of 5-oxoproline.

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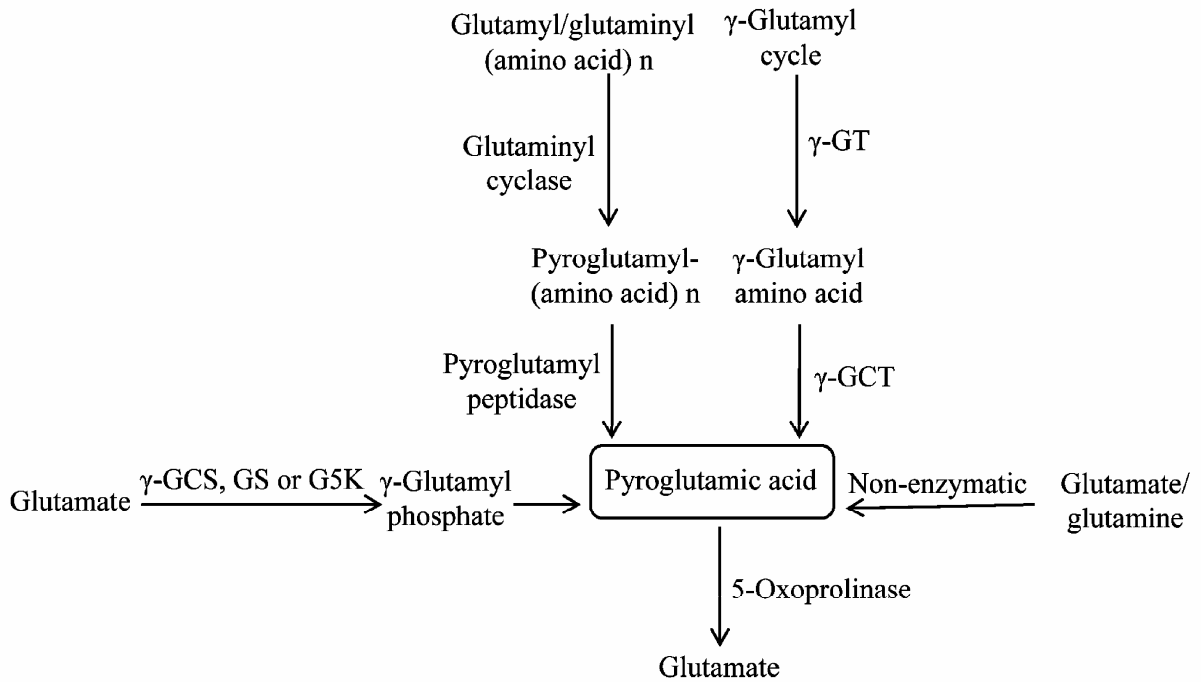

Figure 2. Schematic representation of different routes of 5-oxoproline generation and its utilization. G5K, Glutamate 5-kinase; γ-GT, γ-Glutamyl transpeptidase; γ-GCT, γ-Glutamyl cyclotransferase; γ-GCS, γ-Glutamylcysteine synthetase; GS, Glutamine synthetase.

How does pyroglutamic acid form in living cells?

From the degradation of glutathione

Pyroglutamic acid is an intermediate metabolite of glutathione degradation and has been essentially thought to be produced and utilized by the γ-glutamyl cycle[13] (Figure 2). During glutathione degradation, γ-glutamyl transpeptidase (γ-GT), first acts on glutathione to produce γ-glutamyl amino acids[3]. These γ-glutamyl amino acids are imported inside the cell and are the substrates of γ-glutamyl cyclotransferase (γ-GCT)[2]. γ-GCT acts on γ-glutamyl amino acids to yield pyroglutamic acid and the corresponding amino acids.

The enzyme γ-GCT is widely distributed in animal tissues and has been purified from different mammalian tissues[9,14–16]. The gene encoding human γ-GCT has been identified only recently as C7orf24 (chromosome 7, ORF 24)[10]. This was surprising considering that the protein itself was purified several decades ago. The gene was identified through the sequence of the purified protein, and found to be a small protein of 21 kDa having a fold similar to Butirosin G, the BtrG fold (now BtrG/γ-GCT fold)[17]. The structure of the γ-GCT protein, the active site residues and the mechanism of action of the enzyme were also delineated. Homologues are restricted to metazoa and bacteria[10].

From incomplete reactions following glutamate activation

Pyroglutamic acid has also been found to be produced by glutamate in the presence of γ-GCS, glutamine synthetase and glutamate-5-kinase enzymes[18–20] (Figure 2). The enzyme-bound phosphorylated glutamate is the intermediate in all three enzymatic reactions. In all three cases the activated glutamate is transferred to an acceptor molecule, namely cysteine, ammonia and NADPH respectively. Phosphorylated or activated glutamate is highly unstable and prone to spontaneous cyclization into pyroglutamic acid[21]. If the acceptor molecule is not present or unavailable, spontaneous cyclization of activated glutamate leads to pyroglutamic acid generation (Figure 3). γ-GCS which catalyses the first step of glutathione biosynthesis activates glutamate that may be converted into pyroglutamic acid in the absence of cysteine[19]. Similarly, in methanotrophs it has been proposed that in stress and nitrogen-limiting conditions pyroglutamic acid is generated from glutamate via glutamine synthetase, as found in *in vitro* conditions[22].

From the degradation of proteins containing pyroglutamic acid at the N-terminus

Pyroglutamic acid has been found at the N-terminus of many peptides and proteins playing a role in both the

activity and stability. As pyroglutamic acid cannot be incorporated directly through tRNA[23], the presence of pyroglutamic acid at the N-terminus of these protein is a post-translational event resulting from conversion of N-terminal glutamate and glutamine into pyroglutamic acid by the action of glutaminyl cyclase[24]. This enzyme has been discovered from bacteria, plants and animals. The cleavage of N-terminal pyroglutamic acid from these proteins by the action of pyroglutamyl peptidase discovered in many organisms from bacteria to mammals, generates 5-oxoproline, which forms another important route for pyroglutamic acid formation in the cell[25] (Figure 2).

5-Oxoprolinase: the hydrolysis of pyroglutamic acid to glutamic acid

5-Oxoprolinase hydrolyses pyroglutamic acid to yield glutamate, and is the only enzyme known to act on 5-oxoproline (Figure 2). The glutamate generated can either reenter the γ-glutamyl cycle or be used for other cellular functions. Eukaryotic 5-oxoprolinase is an unusually large dimeric enzyme of about 280 kDa (monomeric size 142 kDa)[26]. It is an ATP-dependant enzyme and has been demonstrated to have an 'actin-like ATPase fold' rather than the P-type ATPase or the Walker ATPase motif seen in many proteins[12]. In the proposed reaction mechanism of the enzyme, put forward by Meister and colleagues in 1970, pyroglutamic acid is first phosphorylated with ATP hydrolysis on the amide carboxyl oxygen to form phosphorylated 5-oxoproline, and the resulting intermediate is subsequently hydrolysed to yield γ-glutamylphosphate. The latter is then be hydrolysed to glutamate and inorganic phosphate[27,28].

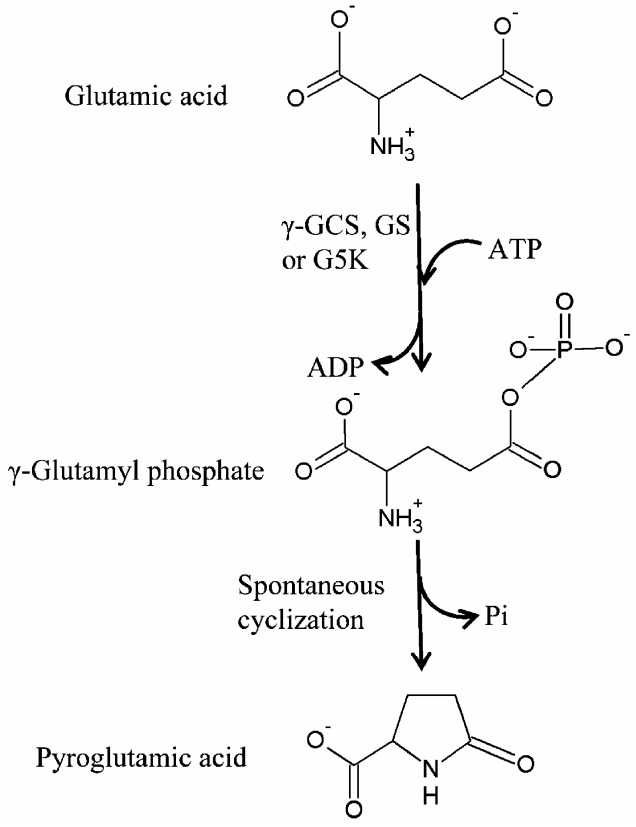

Figure 3. Schematic diagram showing 5-oxoproline generation from partial reactions of γ-GCS, GS and G5K.

The gene encoding mammalian 5-oxoprolinase was identified in mammals in 1996, but heterologous expression and purification of recombinant 5-oxoprolinase was not achieved[11]. Only in 2010, the *Saccharomyces cerevisiae* 5-oxoprolinase, which is similar to the mammalian 5-oxoprolinase, could be recombinantly purified from *S. cerevisiae* (though not from *Escherichia coli*), enabling a more detailed structure–function analysis[12].

The amino terminal part of the protein shows significant similarity with HyuA class of hydantoinase, whereas the carboxy terminus of this open reading frame (ORF) shows similarity with HyuB[11]. The domains have recently been shown to be functionally separable[12]. The similarity of 5-oxoprolinase and hydantoinases may be a consequence of the similarity in their substrates and reactions. The respective substrates, pyroglutamic acid and hydantion, both are five-membered ring compounds and are hydrolysed via their internal amide bond.

A new type of 5-oxoprolinase has been described from bacteria, *Alcaligen feacalis* which has quite different protein and enzymatic characteristics. The protein is relatively small (46 kDa) and is an ATP-independent enzyme[29]. The gene has been identified and the encoded protein does not show similarity with the known 5-oxoprolinase and hydantoinases. Homologues of this gene are only present in few bacterial groups[30].

The assay of pyroglutamic acid and 5-oxoprolinase: a retarding factor in pyroglutamic acid research

Often research progress in a field is limited by simple and convenient assays. This seems precisely to be the case with pyroglutamic acid and 5-oxoprolinases. Mammalian 5-oxoprolinase is a slow-acting enzyme, and needs a sensitive assay method to be studied. Although several methods have been described for assaying 5-oxoprolinase, all of them seem to suffer from some disadvantage.

One of the early methods, that still continues to be used, is based on detection and analysis of radiolabelled glutamate that is formed by the action of 5-oxoprolinase on radiolabelled 5-oxoproline[4]. However, unhydrolysed, radiolabelled pyroglutamic acid is required to be separated from glutamate using ion exchange chromatography. The method is tedious and hazardous, and bound to lose reaction product during the Dowex 50 H^+-based ion

exchange chromatography required to separate it from the unhydrolysed substrate.

Since 5-oxoprolinase is an ATP-dependent enzyme, the inorganic phosphate liberated from the ATPase activity of 5-oxoprolinase has also been exploited for 5-oxoprolinase assay. However, the method is not specific for 5-oxoprolinase and nonspecific ATPases interfere in the assay.

Other methods have included a paper chromatographic method where pyroglutamate was converted to glutamyl hydroxamates and then assayed colorimetrically[31], and a spectrophotometric method based on detection of cysteine generated from 5-oxoprolinase action on OTC (a sulphur-containing analogue of pyroglutamic acid and substrate of 5-oxoprolinase)[32]. However, the methods have lacked sensitivity and in the latter case, does not work with the native substrate.

Glutamate detection has also been attempted as an assay method for 5-oxprolinase. o-Phthaldialdehyde derivatization of glutamate in the presence of thiol, followed by subsequent separation of the product by HPLC has been demonstrated[33]. However, the method is laborious and time-consuming. A sensitive method based on a glutamate detection kit that measures H_2O_2 has also been described. Although sensitive, the method is cumbersome[34].

Thus, despite the plethora of methods, none of them combines sensitivity, ease and rapidity. Recently, we have adapted the simple and extremely sensitive Amplex-Red-based flourimetric glutamate detection method for the purification and assay of 5-oxoprolinase. The Amplex-Red method, based on an assay coupled to H_2O_2 detection, can detect glutamate up to 1 pmol. However, a major difficulty in using this method for 5-oxoprolinase is the strong requirement of 5-oxoprolinase for reducing agents on the one hand, and the extreme sensitivity of the Amplex Red reagent towards reducing agents on the other. By carefully evaluating the sensitivity of both the enzyme and the reagent towards reducing agents, we have been able to devise a convenient assay for the enzyme, that also combines rapidity and sensitivity. The method can also be used for assaying γ-GCT by coupling the reaction with 5-oxoprolinase[12] (A. Kumar and A. K. Bachhawat, unpublished).

Physiological roles of pyroglutamic acid

Pyroglutamic acid in proteins

Many proteins and biologically active peptides exhibit an amino terminus pyroglutamic acid (pGlu) residue (Table 1). The pGlu formation occurs when the N-terminal residue of the protein is either glutamine or glutamate[24]. The formation of pGlu can occur spontaneously, where faster rates are seen with the N-temrinal glutamine. The rate of formation is significantly increased in the presence of an enzyme glutamine cyclase, which can act on both N-terminal glutamine or N-teminal glutamate, although the rates are again faster with the glutamine residue. Structural elements in proteins also influence the reaction rates so that the rate of formation differs with different proteins. The pGlu moiety provides proteins resistance from degradation by amino peptidases. The structural proteins, fibrin, fibrinogen and collagen-like proteins have N-terminal pGlu that protects them from degradation[35]. Several snail conotoxins have also been shown to contain a pGlu at the N-terminal[36,37]. Recently, a simple methodological modification has been described that should facilitate the identification of proteins containing N-terminal pyroglutamate and may lead to the discovery of more proteins with this modification[38].

In addition to its effect on the stability of proteins, the pGlu moiety has an important role in the functionality of the protein or peptide. pGlu is an important determinant of the functionality of many neuropeptides. Examples include[39–42] the thyrotropin releasing hormone (TRH), gastrin, the neuropeptide neurotensin and the human chemokines, MCP1–MCP4. It has been found that pGlu is essential for TRH function, and any alteration or substitution in the pGlu lactam ring significantly decreases both hormone potency and receptor binding ability[43]. Later it was found that TRH binds to its cognate receptor via an interaction between the carbonyl ring of the TRH pGlu moiety and the receptor[44]. MCP-2, a chemotactic protein that activates many immune cells has an N-terminal pGlu that is found to be essential for its chemotactic activity and also protects the protein against protease degradation increasing its stability[42].

The presence of pGlu at the N terminus of some frog RNAase has also been described to be involved in determining its specifity and activity[45].

Almost half the antibodies reported in the literature contain a glutamic acid residue at their N-terminus of light or heavy chain. The formation of pGlu in these antibodies would make them resistant to aminopeptidases and thereby increase their *in vivo* half-life[46]. However, this has not been actually demonstrated. The impact of pGlu on the functionality of the antibody also needs to be carefully evaluated. The formation of pGlu in antibodies and therapeutic proteins is a major irritant in the biotech industry involved in therapeutic proteins, as it results in protein microheterogenity and indicates a lack of process control[47].

In several neurodegenerative disorders, pyroglutamate-containing peptides are involved in pathogenesis. In Alzheimer's disease, Amyloid beta (Aβ) peptide containing N-terminal pGlu is the major peptide in amyloid plaques, and is reported to be neurotoxic and aggregates rapidly[48]. In familial British dementia and familial Danish dementia also, peptides distinct from Aβ are seen, but these too contain an N-terminal pGlu. It is thought that this excessive

Table 1. Some human peptides and proteins with an amino terminal pGlu residue

Protein/peptide	Sequence
TRH	pGlu-His-Pro-NH2
TRH-like peptide (prostate)	pGlu-Glu-Pro-NH2
Anorexigenic peptide	pGlu-His-Gly-OH
Eisenine	pGlu-Glu-Ala-OH
Colon mitosis-inhibitory peptide	pGlu-Glu-His-Gly-OH
Peptide-inhibiting epidermal mitosis	pGlu-Glu-asp-Cys-Lys-OH
Vasoactive polypeptide	pGlu-Val-Pro-Gln-Trp
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH2
GnRH-II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2
Eledoisin	pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH2
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-NH2
Fibrinopeptide B	pGlu-Gly-Val-Asn-Asp-Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg
Gastrin	pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-
Aβ11(pE)–40/42	pGlu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly-Ser-
Aβ3(pE)–40/42-	pGlu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-
Orexin A	pGlu-Pro-Leu-Pro-Asp-Cys-Cys-Arg-Gln-Lys-Thr-Cys-Ser-Cys-Arg-Leu-
Apelin	pGlu-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe

accumulation of pGlu peptides is due to their resistance to aminopeptidases by pGlu[49]. In addition to their proteolytic stability, they also display increased hydrophobicity and decreased solubility, both contributing factors to their deposition as plaques. Biophysical studies have also shown that the pGlu residue in these proteins leads to an increased propensity to aggregate and also cause an increase in inter-fibril associations[50]. The importance of pGlu in pathogenesis of these amyloids is also supported by the fact that the glutaminyl cyclase enzyme largely responsible for the pGlu generation, was found to be upregulated in cortices of individuals with Alzheimer's disease. Moreover, inhibition of glutaminyl cyclase by inhibitors in a mouse model led to decreased level of pGlu-modified Aβ peptides and also attenuated Alzheimer's disease[51]. Surprisingly however, knockouts of glutaminyl cyclase did not show any significant behavioural defects associated with altered TRH and other neuropeptides. Subsequent analysis revealed that these mice were found to still contain pGlu residues, suggesting an alternate route (either spontaneous or by the action of an isozyme of glutaminyl cyclase, iso glutaminyl cyclase)[52].

The functions of pyroglutamic acid as a cellular metabolite

As an analogue of glutamate: The functions of pGlu as a free acid are less clear. However, being a glutamate analogue and a potential precursor and reserve of glutamate, it is strongly linked to all processes involving glutamate. It has been studied as an agonist of glutamate in brain-related research[6]. Pyroglutamic acid is orally active, and can be efficiently transported and can cross the blood–brain barrier, as it is found accumulated in high concentration in the brain after oral administration[53–55]. The transport itself occurs through a Na^+-dependant monocarboxylate transport system[56]. Pyroglutamic acid has been found to prevent scopolamine-induced amnesia in rats, and causes improved learning in age-associated and alcohol-induced memory loss[55]. It can bind glutamate receptors, inhibit glutamate uptake by synaptosome and chronic intrastriatal infusion, and has been shown to cause severe brain cell loss in adult rat[57]. In a study with rats, axotromic retinal ganglion cells were found to survive when treated with pyroglutamic acid, and this was dependant on the non-specific glutamate transporter, EAAT[58]. It also functions as a secondary messenger for the regulation of amino acid availability in brain, since by binding to the amino acid efflux pumps of the blood–brain barrier it stimulates them leading to the efflux of amino acids from the brain[59,60]. A similar function of pyroglutamic acid has also been found in the placenta and mammary glands[61,62].

As a reservoir of glutamate: With pyroglutamate easily being converted to glutamate following hydrolysis, it is apparent that one of the functions might well be as a storage for glutamate, an important metabolite in the cell, with additional key functions in neuronal cells. Labelled pyroglutamic acid leads to the generation of labelled glutamate and γ-aminobutyric acid (GABA)-like neurotransmitters, suggesting that it is actively metabolized in tissues[63].

Recently, because of the considerable quantity of brain glutathione and its rapid turnover, a study was undertaken to investigate whether glutathione can serve as a reservoir of neural glutamate that is the major excitatory transmitter in the central nervous system. The study found that inhibition of 5-oxoprolinase and γ-glutamyl transpeptidase, enzymes that liberate glutamate from glutathione, leads to decrease in glutamate in neuronal cells[64]. These

results indicate that glutathione is not only a reservoir of reduced cysteine, but also functions as a reservoir of glutamate. As 5-oxoproline is an intermediate, the regulatory control at this step remains a possibility.

In the plant *Arabidopsis thaliana*, it was found that deletion of the 5-oxoprolinase gene leads to accumulation of significant amounts of 5-oxoproline, and up to 30% decrease in glutamate level in some tissues like leaves, flowers and siliques, suggesting that pyroglutamic acid is an important precursor and source of glutamate in these organs[65].

The *Bordetella pertussis* periplasmic receptors, DctP6 and DctP7, are soluble components of a non-ATP dependant transport pathway. These receptors belong to a family that is involved in binding different ligands/solutes, followed by their translocation by the membrane component of this transporter. The crystal structures of DctP6 and DctP7 revealed that these receptors strongly bind pyroglutamic acid (with a K_m of 0.3 μM) comparable to the affinity of other anionic transporters to their ligands, and suggesting that it is a true ligand. Despite the strong binding, however, actual uptake of pyroglutamic acid could not be demonstrated[66]. However, the presence of a putative 5-oxoprolinase gene in these organisms, suggests that the presence of pyroglutamic acid-binding transporters is not likely to be an artifact, and pyroglutamic acid may well be a source of glutamate for the cell that can be channelled according to the cellular requirements[66].

As an osmoprotectant and other functions: In the halotolerant methanotroph *Methylobacter alkaliphilum*, pyroglutamic acid was found to accumulate in response to salt stress and function as an osmoprotectant along with ectoin and sucrose, known osmoprotectants. Cells grown in 1 M NaCl are found to accumulate 0.4 M pyroglutamic acid and 1.5 M ectoin. The accumulation of K^+ ion was also found to be equimolar to pyroglutamic acid and was suggested to be the counterion of pyroglutamic acid[67]. High water-binding capacity and the relatively metabolic inert nature of pyroglutamic acid makes it a compatible solute and osmoprotectant. In this context it is interesting that thermophilic lactobacilli used as starters in the ripening of Italian cheeses (Grana Padano and Parmigiano Reggiano), cause the accumulation of significant levels of pyroglutamic acid in the cheese (up to 0.5 g/100 g). The formation is thought to be enzymatic owing to the exclusive formation of L-pyroglutamic acid, but why these thermophilic lactobacilli accumulate such high levels of pyroglutamtic acid is not known[68]. In a metabolome study of the gut microflora in patients affected with Irritable bowel syndrome, high levels of pyroglutamic acid were detected in addition to a few other metabolites, and these were also correlated with increased presence of lactobacilli and clostridium. The reason for these increased levels was however unclear[69]. In mammals, the level of pyroglutamic acid and pyroglutamic acid-generating enzyme, γ-GCT, is comparatively high in skin, and it appears that it might function as a natural moisturizer[70,71]. The *de novo* synthesis of pyroglutamic acid in response to osmotic stress is proposed to occur by a constitutive enzyme glutamine synthetase, that cyclizes the glutamate into pyroglutamic acid in the absence of ammonia[22].

Pyroglutamic acid has also been shown to have an antidiabetic effect in type 2 diabetes, as seen from feeding experiments with rats and mice[72], which is suggested to occur through modification of glucose and lipid metabolism. The ligand of the Angiotensin-like G-protein-coupled receptor includes the Apelin peptide hormone. This hormone has many active forms, including Apelin-13, a pyroglutamylated peptide[73]. These peptides have an important role in cardiovascular disorders, insulin resistance and obesity. Whether these two aspects, i.e. the pyroglutamic acid and hormone action are linked is, however, not clear.

5-Oxoprolinuria: defects in the γ-glutamyl cycle, acetaminophen-induced metabolic acidosis and cystinosis

5-Oxoprolinuria is the secretion of high levels of pyroglutamic acid in the urine. Under normal conditions, pyroglutamic acid levels vary from 0.5 to 5 mg/day in excretion. However, in diseased conditions it increases up to 50 g/day, indicating large-scale pyroglutamic acid secretion in the urine[74]. A variety of different pathological conditions can cause 5-oxoprolinuria (Figure 4). How defects in such varied pathways lead to the secretion of pyroglutamic acid is intriguing.

5-Oxoprolinuria as a result of inherited errors, is due to metabolic defect in either of the two enzymes of the γ-glutamyl cycle, glutathione synthetase or 5-oxoprolinase.

Deficiencies in the glutathione synthetase step is found in majority of the reported cases of 5-oxoprolinuria[74,75]. The enzyme glutathione synthetase is responsible for glutathione synthesis from its precursor γ-glu-cys[76]. Error at this step leads to reduced level of glutathione and accumulation of γ-glu-cys, a substrate of γ-GCT, which generates pyroglutamic acid from γ-glu-cys[75]. The first enzyme of the glutathione biosynthesis, γ-glutamylcysteine synthetase, is under the feedback regulation of glutathione[77]. Lower glutathione levels because of a defect in the glutathione synthetase step relieves the γ-glutamylcysteine synthetase enzyme from feedback inhibition, thereby producing more γ-glu-cys in this condition. This further leads to overproduction of pyroglutamic acid that surpasses the capacity of 5-oxoprolinase (a sluggish enzyme) and leads to increased pyroglutamic acid in body fluid, blood, cerebrospinal fluid and massive urinary excretion of pyroglutamic acid[78]. This condition also leads to severe metabolic acidosis, hemolytic anaemia

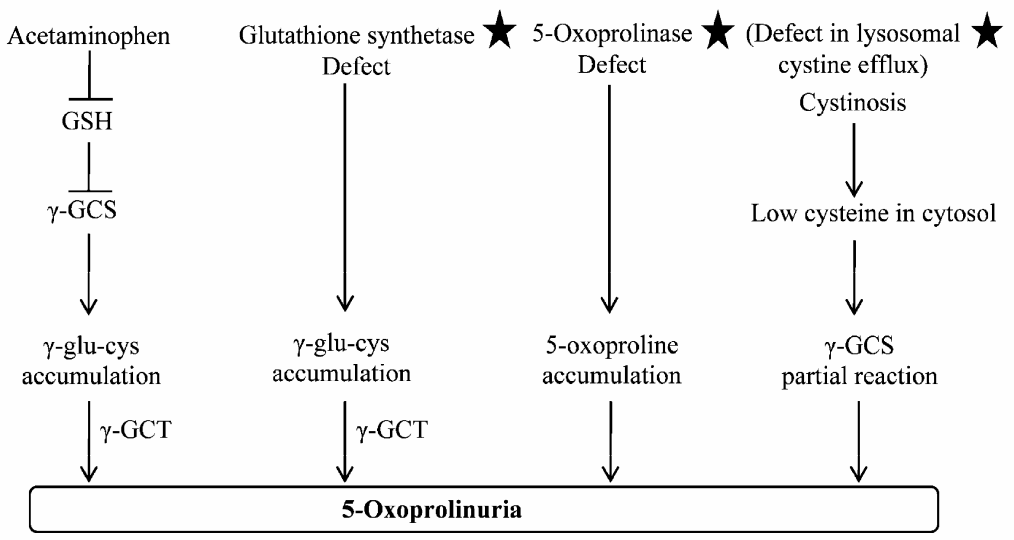

Figure 4. Schematic presentation of involvement of γ-glutamyl cycle, acetaminophen and cystinosis in 5-oxoprolinuria.

and central nervous system dysfunction[79]. More than 70 patients have been reported in more than 50 families worldwide, having 5-oxoprolinuria because of a defect in the glutathione synthetase enzyme[74]. No patients have been reported from India.

A defect in the 5-oxoprolinase enzyme has also been found to result in 5-oxoprolinuria although this is not as prevalent and severe as the γ-glutamylcysteine synthetase step-mediated disease[80,81]. 5-Oxoprolinase deficiency is an extremely rare autosomal recessive disease. Only eight patients from six families have been reported in the literature. All the patients were diagnosed based on high pyroglutamic acid in the urine, although they had normal glutathione synthetase cellular enzyme levels and absence of metabolic acidemia[74].

Acetaminophen-mediated acquired 5-oxoprolinuria is also being observed with increasing frequency. It is well known that chronic ingestion of acetaminophen depletes glutathione reserve through its intermediate metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI). NAPQI irreversibly conjugates with glutathione and relieves the γ-glutamylcysteine synthetase from feedback inhibitions of glutathione. This leads to accumulation of γ-glu-cys and thus pyroglutamic acid[82,83]. The excess of pyroglutamic acid that forms 5-oxoprolinuria leads to metabolic acidosis and increased anion gap (difference in the measured cations and the measured anions in serum, plasma or urine). Interestingly though, acetaminophen-induced metabolic acidosis seems to be exclusively seen in women patients, suggesting perhaps distinct sex-dependant differences in some of these metabolic enzymes.

5-Oxoprolinuria has also been observed in patients of cystinosis. This is an inherited disease caused by a defect in the lysosomal cystine transporter (CTNS). It is characterized by sequestration of cystine in the lysosome, and leads to renal proximal tubular dysfunction[84]. A key aspect in the pathophysiology of the disease is the reported ATP depletion that occurs in cystinotic cells, a depletion that is not a consequence of decreased synthesis[85]. An observation in patients with nephropathic cystinosis, is that they contain almost 60-fold higher levels of pyroglutamic acid in their urine[86]. The excess pyroglutamic acid has been explained as being due to a defect in the γ-glutamyl cycle, but how this could lead to pyroglutamic acid secretion has not been explained[87]. We recently hypothesized that as a consequence of the defective cystinosin gene, the cell is unable to get sufficient levels of cytosolic cysteine[88]. This can result in the formation of pyroglutamic acid through partial reaction of the γ-GCS enzyme as it is known that if γ-GCS the enzyme, fails to find the acceptor (cysteine) for γ-glutamyl phosphate (an intermediate of γ-GCS reaction), the activated γ-glutamyl phosphate can autocyclize to form pyroglutamic acid[19]. The pyroglutamic acid is again hydrolysed to glutamate at the expense of ATP[4]. This leads to a futile cycle resulting in ATP depletion. The sluggish nature of the 5-oxoprolinase enzyme prevents all the pyroglutamic acid from being removed from the system, thereby also explaining the accumulation of pyroglutamic acid[88].

Future directions

Although a role for pyroglutamic acid beyond the mere intermediate metabolite role initially assigned to it has clearly emerged over the years, we still have a poor understanding of the role of pyroglutamic acid in cellular physiology. Recent research in plants as well as in different neuronal cells has clearly indicated a glutamate reservoir-like role of pyroglutamic acid and its parent

molecule, glutathione. The regulation of this reservoir needs to be better understood in different cells. However, the cellular role does not seem limited to this, since pyroglutamic acid itself has been shown to influence different cellular processes. The observation that 5-oxoprolinases are found in prokaryotic organisms that do not produce glutathione also suggests a role beyond an intermediate in glutathione degradation. An aspect that needs to be examined in this context is the possible role of pyroglutamic acid as an osmoprotectant in some microbial cells, and the large amount of pyroglutamic acid found in the skin.

5-Oxoprolinuria is not a common disorder. However, the incidence of acetaminophen-induced metabolic acidosis and its effect on causing 5-oxoprolinuria is increasing. Understanding the precise mechanisms by which this occurs, and validating the different hypotheses of pyroglutamic acid secretion in cystinosis and acetaminophen-induced metabolic acidosis would give rise to a better understanding of the pathophysiology of these disorders.

A limitation in pyroglutamic acid research over the years, in the laboratory and the clinic, has been the difficulty of pyroglutamic acid assay. It might also explain the fact that no patients showing pyroglutamic acid excretion in the urine have been reported from India. Although one Indian patient has been reported with cystinosis, the pyroglutamic acid levels were not reported. Further improvement in assays for routine assaying of pyroglutamic acid in hospitals is a necessity.

Regulatory studies on γ-glutamyl cyclotransferases and 5-oxoprolinases, the enzymes that principally make and remove pyroglutamic acid, have been completely lacking. An understanding of these aspects is important if we aim to understand the role of these enzymes and pyroglutamic acid, and their contribution to the different cellular processes.

A lot of recent work has focused on N-terminal pyroglutamate found in certain proteins, and as the appreciation of this post-translational modification increases, and the regulatory role in addition to the structural and role in activity that they might be playing, one can only hope that we will gradually come to a better appreciation and understanding on this apparently ubiquitous metabolite. The role of this modification in protein has largely been ignored till now, as it has most often been viewed dismissively as only an irritant in protein sequencing protocols. However, the importance of controlling this modification in therapeutic proteins as well as the presence of this modification on a variety of neuropeptides, and on the peptides responsible for Alzheimer's disease and familial dementia have created greater interest to understand this modification.

Indeed, pyroglutamic acid is still largely a forgotten metabolite, but with the recent revival in interest, one can only hope that the scenario with respect to our understanding of this interesting metabolite, is changing.

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ACKNOWLEDGEMENTS. A.K. acknowledges support from the Council of Scientific and Industrial Research, New Delhi. This work was supported in part by a Grant-in-Aid Project received from the Department of Science and Technology, New Delhi.