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Pyrrolidone Carboxyl Peptidase (Pcp): An Enzyme That Removes Pyroglutamic Acid (pGlu) From pGlu-Peptides and pGlu-Proteins

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ABSTRACT **Pyrrolidone carboxyl peptidase (EC 3.4.11.8) is an exopeptidase commonly called PYRase, which hydrolytically removes the pGlu from pGlu-peptides or pGlu-proteins.**

pGlu also known as pyrrolidone carboxylic acid may occur naturally by an enzymatic procedure or may occur as an artifact in proteins or peptides. The enzymatic synthesis of pGlu suggests that this residue may have important biological and physiological functions. Several studies are consistent with this supposition.

PYRase has been found in a variety of bacteria, and in plant, animal, and human tissues. For over two decades, biochemical and enzymatic properties of PYRase have been investigated. At least two classes of PYRase have been characterized. The first one includes the bacterial and animal type I PYRases and the second one the animal type II and serum PYRases. Enzymes from these two classes present differences in their molecular weight and in their enzymatic properties.

Recently, the genes of PYRases from four bacteria have been cloned and characterized, allowing the study of the primary structure of these enzymes, and their over-expression in heterelogous organisms. Comparison of the primary structure of these enzymes revealed striking homologies.

Type I PYRases and bacterial PYRases are generally soluble enzymes, whereas type II PYRases are membrane-bound enzymes. PYRase II appears to play as important a physiological role as other neuropeptide degrading enzymes. However, the role of type I and bacterial PYRases remains unclear.

The primary application of PYRase has been its utilization for some protein or peptide sequencing. Development of chromogenic substrates for this enzyme has allowed its use in bacterial diagnosis.

Key words: enzymology, protein structure, biochemical properties, gene characterization, bacterial diagnosis

INTRODUCTION

Aminopeptidases (E.C. 3.4.11.1–14) are N-terminal exo-peptidases of great importance in biology and medicine because of their role in protein and peptide metabolism. These enzymes are involved in protein modification and, in particular, in protein and peptide catabolism. They exhibit specificity for the hydrolysis of peptide bonds that contain the aminoacyl residue present at the N-terminus of polypeptides. Because of this activity, these enzymes have proved useful in protein sequencing, in other protein chemistries, and also in the diagnosis of bacterial strains.[1] The pyrrolidone carboxyl peptidase, L-pyroglutamyl-peptide hydrolase, or PYRase (E.C. 3.4.11.8), represents one class of amino peptidases. This peptidase, also known as 5-oxoprolyl-peptidase (E.C. 3.4.19.3), pyroglutamate aminopeptidase,[2,3] pyroglutamyl aminopeptidase,[4] pyrrolidonyl peptidase,[5,6] pyrrolidonecarboxylate peptidase,[7,8] and pyrrolidonecarboxylyl peptidase,[9,10] specifically removes the L-pyroglutamyl residue from the amino-terminus of polypeptides by hydrolysis (Fig. 1).

Since the discovery of PYRase over two decades ago,[5] its activity has been demonstrated in bacteria, plant, animal, and human tissues.[6,11] The biochemical and enzymatic properties of PYRase have been investigated, and the enzyme has more recently been implicated in hormone regulation and in metabolism by neuronal cells in mammalian systems.[12–14] Genes for the bacterial PYRases have been cloned and sequenced, allowing for their over-expression and studies on primary structure.

In view of the importance of PYRase in protein sequencing, protein chemistries, bacteriology, and hormone regulation, and considering the increased interest in this class of enzymes, we have under-

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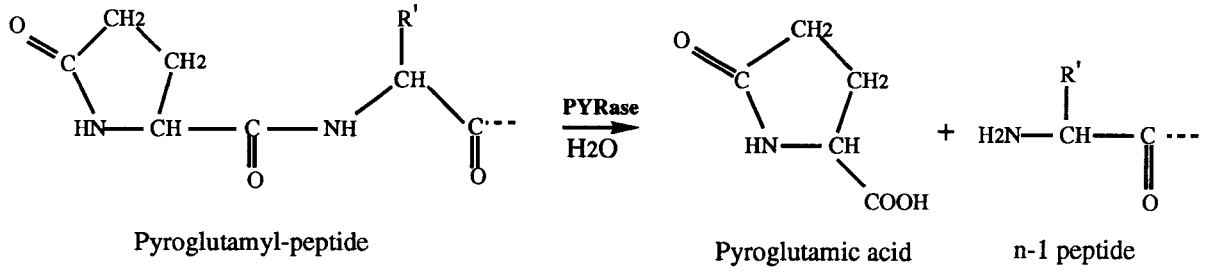


Fig. 1. Hydrolysis of pGlu from the N-terminus of L-pGlu-proteins or L-pGlu-peptides by PYRase.

taken to review what is known about the PYRases. We begin our review by providing insight into the enzymology of pyroglutamic acid and of its biological occurrence. Next, we discuss the biochemical and enzymatic properties of the PYRases, reviewing the recent characterization of several PYRase genes, and include an analysis of their primary structure. Finally we discuss the possible physiological role of PYRases and how these enzymes are of use in practical applications.

ENZYMOLOGY AND OCCURRENCE OF PYROGLUTAMIC ACID

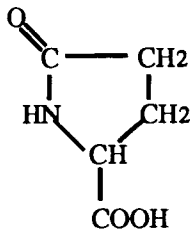
pGlu (5-oxo-L-proline), also known as pyrrolidone carboxylic acid, was first described by Haitinger[15] as a glutamic acid derivative that lacked a molecule of water (Fig. 2). Evidence for the nonenzymatic formation of pyroglutamic acid has been reported by several workers.[16–18] The compound has been derived from glutamic acid, glutamine, γ-esters of glutamic acid, γ-diesters of glutamic acid, γ-glutamyl-peptide, and γ-glutamyl hydroxomate (reviewed in ref. 19). Menozzi and Appiani[20,21] have confirmed the structure of pyroglutamic acid by showing that it could be generated by heating glutamic acid to 180°–190°C.

The enzymatic synthesis of pyroglutamic acid was demonstrated when enzymes were discovered that could convert D-glutamic acid to D-pyrrolidone carboxylic acid. The first indication of this was from a study by Wilson and Koepee,[22] who showed that intraperitoneal and subcutaneous administration of D-glutamate to the rat led to the overproduction of D-pyrrolidone carboxylic acid. These workers also went on to show that free D-glutamic acid could be converted to D-pyrrolidone carboxylic acid by extracts of rat liver and kidney.[22] The conversion of D-glutamic acid to D-pyrrolidone carboxylic acid has been shown to occur by an intramolecular acylation that is catalyzed by D-glutamic acid cyclotransferase; this enzyme has been partially purified from animal tissue and partially characterized.[23] Orlowski and Meister[19] have suggested that the role of D-glutamic acid cyclotransferase may be to detoxify D-glutamate, which can be formed by the intestinal flora, or introduced through diet. Akita et al.[24] have described a reaction in *Pseudomonas cruciviae* that involves the conversion of L-glutamate to L-pyrrolidone carboxylic acid by L-glutamic acid cyclotransferase. This enzyme may also be present in *Streptococcus bovis*.[25]

The enzymatic conversion of glutamate to pyroglutamic acid has also been shown to occur through a slow reaction catalyzed by glutamine synthetase in the presence of ATP and Mg^{2+}.[26] γ-Glutamylcysteine synthetase has been shown to catalyze this reaction too in the presence of ATP, L-cysteine, and Mg^{2+}.[19]

pGlu may occur as an artifact of protein or peptide hydrolysis during the liberation of the N-terminal glutamyl or glutaminyl residue.[5,27] An example of this may occur in the formation of "bitter" peptides in cultured dairy products. Sullivan and Jago[28] suggested that "bitterness" might be due to the liberation of pGlu during N-terminal proteolysis of a hydrophobic peptide that is derived from casein. The artifactual occurrence of pyroglutamic acid also presents a problem encountered in protein sequencing; N-terminal pGlu can be formed in the preparation of peptides from proteins in sequencing.[16,17,29–31]

pGlu may also occur naturally in proteins and peptides as a result of a catalyzed cyclization of the N-terminal glutamyl or glutaminyl residue. For example, L-glutamyl transferase[32,33] and γ-glutamyl transferase (in combination with γ-glutamyl cyclotransferase)[34] have been shown to catalyze the formation of L-pyrrolidone carboxylic acid and pyrrolidone carboxyl peptides from glutamine and glutaminyl peptides, respectively. Enzymes that catalyze the formation of glutaminyl from pyroglutamyl-peptides, however, were not discovered until later because of technical difficulties encountered in the study of these relatively unstable glutaminyl-peptides.[35] It was proposed by Richter et al.[36] that cyclization, particularly of the N-terminal glutamine residue, may occur spontaneously. Busby and co-workers,[35] however, have demonstrated that spontaneous cyclization is a very slow process in vivo. Moreover, these authors have characterized enzymes in mammalian tissues that can convert



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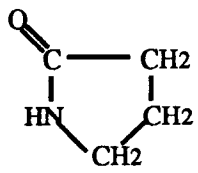

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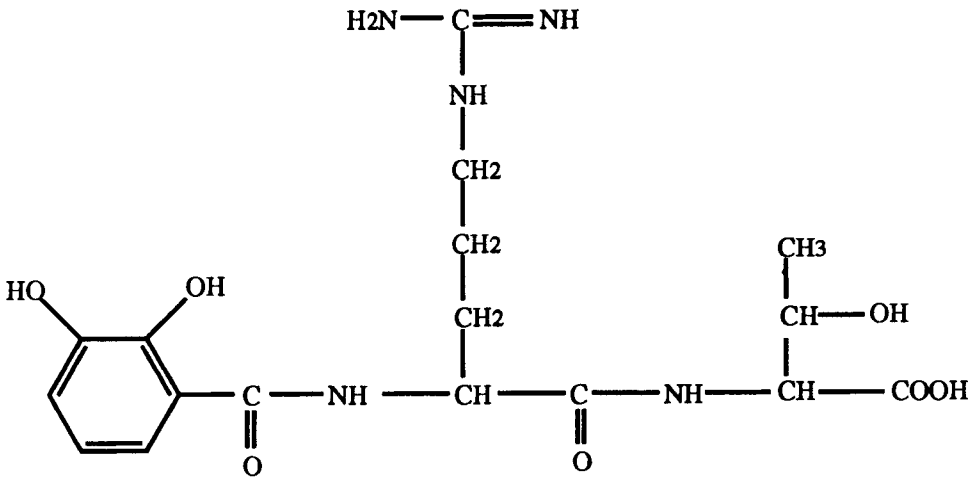

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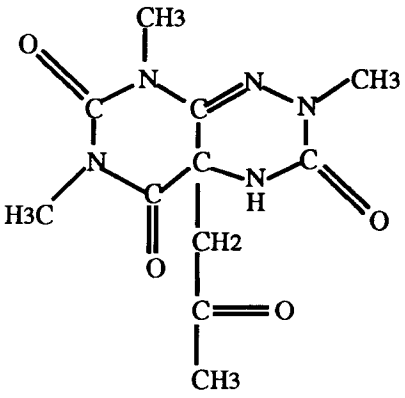

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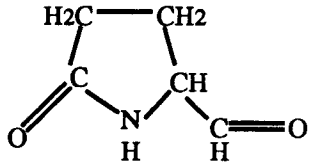


(V)

Fig. 2. Structures of pyroglutamic acid (I), the substrate for PYRase, and some inhibitors of PYRase: 2-pyrrolidone (II), benarthin (III), pyrizinostatin (IV), and 5-oxoprolinal (V).

glutaminyl-peptides into pyroglutamyl-peptides. Fischer and Spiess[37] have also identified a mammalian glutaminyl cyclase capable of converting glutaminyl peptides into pyroglutamyl-peptides. Elsewhere, it has been shown that γ-glutamyl-amino acids can be converted into pyrrolidone carboxylic acid and amino acids by a γ-glutamyl cyclotransferase.[34,38,39]

The enzymatic synthesis of pGlu suggests that this residue may have important biological and physiological functions. Consistent with this is the observation that the N-terminus of many proteins and bioactive peptides ends in pGlu (see Table I) and that the activity of some bioactive peptides is usually associated with the presence of an N-terminal pGlu.[77,78] Moreover, as a free acid, pyroglutamate has been found in the tissues of patients with Hawkinsinuria disease, which is characterized by a disorder of tyrosine metabolism.[79] An increased level of free L-pyroglutamic acid has been shown in the plasma of patients with Huntington's disease.[80] Creer and co-workers[81] have also observed an increased level of pyroglutamate in the plasma of yet other patients.

The pharmacological properties of pGlu residue have been described. Pyroglutamate has been shown to prevent scopolamine-induced amnesia and electroconvulsive shock in the rat.[82] Also, it has been shown to improve learning and age-associated memory loss.[83]

Up until now it has not been clear whether the N-terminal pyroglutamyl residue arises by a post-synthesis catalysis of glutamyl or glutaminyl peptides, or by incorporation as a free residue at the N-terminus of the polypeptides. Both processes may be possible, although current evidence only supports the first possibility. The N-terminal residue in polypeptides may undergo a post-translational modification[35] in which any of the aforementioned enzymes may be involved. There is no evidence as yet to support the N-terminal addition of a pGlu residue. One might conclude that the occurrence of pGlu at the N-terminus of polypeptides may minimize their degradation, or provide them with a particular function. It is noteworthy that, in addition to the enzymatic ATP-dependent decyclization of pyroglutamic acid by a 5-oxoprolinase,[84–88] pyrrolidone carboxyl peptidase may be involved in such peptide level regulation in tissues or cells.

BIOCHEMICAL AND ENZYMATIC PROPERTIES OF PYRase

A number of PYRases have been characterized from bacteria and animals.[2–8,10,12,14,26,89–104] A summary of the properties of these PYRases is provided in Table II. Among the bacterial enzymes, the *Streptococcus pyogenes,*[91] *Bacillus subtilis,*[92] *B. amyloliquefaciens,*[106] and *Pseudomonas fluorescens*[93] PYRases have been purified to homogeneity after overexpression in host cells. The overexpressed *B. amyloliquefaciens* enzyme has been crystallized[106] and therefore a knowledge of PYRase structure is imminent.

Molecular weight (MW) determinations of the bacterial PYRases under native and denaturing conditions reveal that they are similar in size. Determinations under denaturing conditions indicate a mean MW for the bacterial enzyme of about 25,000. Native gel conditions, on the other hand, indicate more variability in size and show an MW range from 50,000 to 91,000. Tsuru et al.[90] have proposed that the *B. amyloliquefaciens* enzyme was probably a trimer. On the other hand, workers from his laboratory[106] suggested more recently that the overexpressed *B. amyloliquefaciens* enzyme was probably a dimer, rather than a trimer or tetramer, as suggested by the isolated native enzyme. These observations suggest that the expressed enzyme is different from the native enzyme.

Other studies indicate that the *S. pyogenes* and *B. subtilis* enzymes are probably tetramers.[91,92] The subunit MW of the *Streptococcus faecium* PYRase has been estimated at 42,000,[7] i.e., an MW almost twice that of the other bacterial enzymes. Interestingly, antibodies against the PYRase of *S. pyogenes* reacted with two protein bands in a Western blot of crude cell extract.[92] As this had never before been reported in bacteria, it was suggested that *S. pyogenes* possessed at least two different PYRases, the larger of which, having a MW of ≈ 40,000, approximated the size of the *S. faecium* enzyme. It would also be of interest to determine the MW of the *S. faecium* enzyme since it has been reported to be a dimer of 24,000 subunits, as suggested by Sullivan et al.,[7] rather than a tetramer, possibly like most other bacterial enzymes.

It must be noted that the suggested differences in multimeric states may be a reflect of the different techniques used to determine MW. Thus an accurate method of MW determination, such as electro-spray mass spectoscopy, analytical ultracentrifugation, or light scattering, may help to resolve this multimer issue. Nevertheless, variability in PYRase size, particularly as seen in the genus *Streptococcus,* indicates a structural complexity in this class of proteins and, in any event, it appears that the bacterial PYRases are oligomeric enzymes.

Studies on eukaryotic PYRases have been done primarily in mammals, and MW determinations have been made mostly by gel filtration (Table II). Two types of PYRases have been identified in animal systems: a soluble PYRase, whose MW is similar to that of the bacterial PYRase and that has sometimes been called pyroglutamyl peptidase I, and a much larger PYRase of between 230,000 and 280,000 MW, which is membrane associated and has been called pyroglutamyl peptidase II.[4,11,98,102] Thus it appears that in animals there are at least

TABLE I. Some Proteins or Peptides With N-Terminal Pyroglutamic Acid Residue

Peptide or protein	Sequence	Reference
Fastigiatine	pGlu-Glu-GlnOH	40
Eledoisin	pGlu-Pro-Ser-Lys-	41
Fibrinopeptides B		
Human	pGlu-Gly-Val-Asp(NH_2)-	42
Ox	pGlu-Phe-Pro-Thr-Asp-	42
Reindeer	pGlu-Leu-Ala-Asp-	42
Bovine	pGlu-Phe-Pro-Thr-Asp-	43
Physalaemin	pGlu-Ala-Asp(OH)-Pro-	44
Peptides from snake venoms	pGlu-Asp(NH_2)-Trp	45
	pGlu-Glu(NH_2)-Trp	45
H_2-proteinase from the venom of the Habu snake	pGlu-Arg-Phe-Pro-Gln-	46
Heavy chains of rabbit IgG	pGlu-Ser-Val-Glu-Glu-	47
	pGlu-Ser-Leu-Glu	47
	pGlu-Glu(NH_2)	47
Eisenine	pGlu-Glu-AlaOH	48
Neurotensin	pGlu-Leu-Tyr-Glu-Asn-	49
TRH.	pGlu-His-Pro(NH_2)	50
TRH-like peptide		
Prostate	pGlu-Glu-Pro-NH_2	51
Alfalfa	pGlu-Tyr-Pro-NH_2	52
Gastrin		
Man	pGlu-Gly-Pro-Trp-Leu-	53
Hog	pGlu-Gly-Pro-Trp-Met-	53
Vasoactive polypeptide	pGlu-Val-Pro-Gln-Trp-	54
Heavy chain from human pathological IgG	pGlu-Val-Thr-	55
Heavy chain of human γG immunoglobulin	pGlu-Val-Gln-Leu-	56
Mouse λ chains	pGlu-Ala-Val-Val-	57
λ-Type Bence-Jones proteins		
Type BO	pGlu-Ser-Ala-Leu-	58
Type Ha	pGlu-Ser-Val-Leu-	58
α2-CB1 of rat skin collagen	pGlu-Tyr-Ser-Asp-Lys-	59
Human apoLp-Gln-II	pGlu-Ala-Lys-Glu-Pro-	60
Thymic factor from pig serum	pGlu-Ala-lys-Ser-Gln-	61
FMRFamide-like peptide from *Helix*	pGlu-Asp-Pro-Phe-Leu-	62
Hypertrehalosaemic neuropeptide	pGlu-Val-Asn-Phe-Ser-	63
Peptide inhibiting epidermal mitosis	pGlu-Glu-asp-Cys-Lys(OH)	64
Colon mitosis inhibitory peptide	pGlu-Glu-His-Gly(OH)	65
Caerulein	pGlu-Gln-Asp-Tyr(SO_3H)-	66
Levitide	pGlu-Gly-Met-Ile-Gly-Thr-	67
Neuropeptide from pennatulid	pGlu-Gly-Arg-Phe-amide	68
Appetite-depressing peptide	pGlu-His-Gly	69
Trypsin inhibitor from bottle gourd	pGlu-Arg-Arg-Cys-Pro-	70
Molt-inhibiting and hyperglycemic peptide from lobster	pGlu-Val-Phe-Asp-Gln-	71
Porcine pancreatic spasmolytic polypeptide	pGlu-Lys-Pro-Ala-Ala-	72
Heavy chains of rabbit anti-hapten antibodies	pGlu-Ser-Leu-Glu-Glu-	73
	pGlu-Ser-Val-Glu-Glu-	73
Gonadotropin-releasing hormones (GnRH) from dogfish brain	pGlu-His-Trp-Ser-His-	74
Human monocyte chemoattractant	pGlu-Pro-Asp-Ala-Ile-	75
Growth hormone from Tilapia	pGlu-Gln-Ile-Thr-Asp-	76

two genes encoding PYRases of similar enzymatic activity. This, however, awaits confirmation through the cloning and characterization of the animal PYRase genes. The oligomeric structure of the animal PYRases is unknown. In view of their MW, some PYRases, such as the human skeletal muscle, human cerebral cortex, and human kidney enzymes, may be monomers.

The enzymatic properties of the PYRases have been studied in order to determine optimal reaction conditions and reaction kinetics. Bacterial PYRases generally show optimal activity at pH 7 to 9.[5–8,91] This observed activity, however, may be influenced by how the enzyme is prepared, since, for example, the optimal pH of the *B. subtilis* enzyme is 7 for the purified, expressed enzyme and 8–9 for the enzyme

TABLE II. PYRases From Different Organisms*

Source	Purification	Optimum pH	Apparent MW	Subunits MW	Gene characterization	Localization	References
P. fluorescens	+	6.5–8.5 or 8	41,000	22,500	+	Intracellular	5, 9, 93
B. subtilis	+	8–9 or 7	91,000	24,000	+	Intracellular	6, 92, 105
B. amyloliquefaciens	+	7–8 or 6.5	72,000 or 51,000	24,000	+	Intracellular	90, 106
S. faecium	+	7.6	ND	42,000	–	Intracellular?	7
S. pyogenes	+	7.0	85,000	23,500	+	Intracellular	91, 104
K. cloacae	+	7.0	74,000	ND	–	Intracellular?, "particulate"	8
Bovine pituitary	+–	7.3	25,000	25,000?	–	Intracellular	96
Guinea pig brain	+–	ND	24,000	24,000?	–	Intracellular	107
Human skeletal muscle	+–	8.5	22,000	22,000?	–	Intracellular	4
Human cerebral cortex	+–	8.5	23,000	23,000?	–	Intracellular	98
Human kidney	+–	8.0	22,000	22,000?	–	Intracellular	102
Rat brain	–	8–8.4	55–65,000	ND	–	Intracellular	108, 109
Rabbit brain	+	7.0–7.5	230,000	ND	–	Extracellular, membrane-bound	99
Guinea pig brain	+–	7.4	230,000	ND	–	Extracellular, membrane-bound	3, 110
Rat liver	+–	ND	240,000	ND	–	Extracellular, membrane-bound	100
Porcine serum	+–	6.5–8.0	260,000	ND	–		12
Rat serum	+–	6.5–8.0	260,000	ND	–		2

*Purification: +, purified to homogeneity; +–, partially purified; –, not purified.
Gene characterization: +, characterized; –, not characterized. ND, not determined. Presence of ? means a supposition.

extracted directly from the organism (cf. refs. 92 and 6); this is a further indication that the cloned, heterologously expressed PYRase differs from the native enzyme. The optimal pH for the animal PYRases has been reported to occur between pH 6.5 and 8.5.[2,4,99] It is difficult to compare directly the activity of the different PYRases because different substrates have been used to determine Michaelis-Menten constants. Nevertheless, it appears that the PYRases exhibit Michaelis-Menten-type kinetics, rather than allosteric-type kinetics, which might be expected for this apparently oligomeric enzyme. It should be added that in reference to K_m, which generally falls between 0.2 and 2 mM (in the case of bacterial enzymes), the affinity of the PYRases for substrate is relatively low.

PYRases, except for type II, belong to the arylamidase group of enzymes, since they are capable of hydrolyzing the peptide bond of the synthetic chromogenic substrate L-pGlu-β-naphthylamide.[111] Other synthetic chromogenic substrates, such as L-pGlu-*p*-nitoanilide and L-pGlu-4-methylcoumarinylamide, are also hydrolyzed by PYRase.[112] Specificity studies have been carried out on L-pGlu-L-amino acids; however, no general rule concerning PYRase activity for these substrates could be arrived at by these studies. From one PYRase to another, the rate of hydrolysis is seen to depend on the amino acid adjacent to the pGlu residue, but this may vary considerably. For instance, it has been shown that the enzyme from *Klebsiella cloacae* was capable of splitting pGlu-proline, but that this compound was not hydrolyzed by the PYRases of *P. fluorescens*,[27] *B. amyloliquefaciens*,[113] and bovine pituitary.[96] PYRases appear to have a strict specificity for L-pGlu, since no enzyme activity is observed when this residue is replaced, or substituted for by D-pGlu.

The activity of the PYRases on biologically active proteins has been investigated. PYRase has been shown to remove the N-terminal pGlu from thyrotropin-releasing hormone (TRH), and from the bradikynin potentiator neurotensin, leuteinizing hormone-releasing hormone (LHRH), litorin, and physalaemin.[113] An interesting feature of type II PYRase from animals is that it appears to have a unique substrate specificity. The most preferred substrate for this enzyme is TRH and TRH analogs such as pGlu-HisPro-NA.[12,99] The first position (P1 position) favors the pyroglutamyl group, and a histidine residue in the P1′ position appears to be essential. This specificity, in contrast, is not observed for the type I PYRases and the bacterial PYRases.

Stability studies have been carried out mainly on the bacterial PYRases; little is known about the stability of animal PYRases. The bacterial enzyme is heat sensitive; it is rapidly inactivated at temperatures above 50°C.[6–8,90] The membrane-associated and serum PYRases of animals may be stable. Except for the type II PYRases, PYRases are generally sulfhydryl-dependent enzymes that can be poisoned by iodoacetamide or other sulfhydryl-blocking re-

agents. Thus, it is necessary to protect this enzyme with reducing reagents such as β-mercaptoethanol during purification and storage. The enzyme can also be stabilized by a noncompetitive substrate analog such as 2-pyrrolidone (Fig. 2); activity is recovered by subsequent removal of the analog by dialysis. As stated above, type II PYRase is not inhibited by sulfhydryl-blocking reagents. This enzyme is, however, inhibited by chelating agents such as EDTA, 8-hydroxyquinoline, and 1,10-phenantroline, and by dithiothreitol (contrary to PYRase I).[12,14,99,100]

Studies with inhibitors of protease activity have led to the conclusion that type II PYRase is a metalloenzyme.[99,114] In contrast, maximum activity of bacterial PYRases and type I PYRases are generally obtained in the absence of divalent ions.[4,6,90,91] Indeed, trace amounts of Hg^{2+}, for example, inhibit these enzymes. The effect of other divalent ions is variable and depends on the enzyme; these other divalent ions typically have a lesser effect than Hg^{2+} or no effect. In some cases, as for the *K. cloacae* peptidase, Ca^{2+} ions can increase enzyme activity.[8] These differences in the effect of divalent ions may be explained by their interaction with the PYRases, especially with respect to the effect these cations may have on enzyme conformation. To our knowledge no work has been done in this area.

In addition to the agents cited above, other compounds have also been shown to inhibit PYRase activity. In particular, O-phenantroline and antipain,[4] N-ethylmaleimide, puromycin and bestatin,[102] Na-tetrathionate, PCMB and N-bromosuccinimide,[90] bacitracin, L-pyroglutamyl chloromethyl ketone and (Z)-pyroglutamyl diazomethyl ketone,[115,116] 5-oxoprolinal (Fig. 2),[117] and benarthin, pyrizinostatin (Fig. 2), and benzamidine[118–122] have been shown to be possible inhibitors of PYRase. The mode of inhibition by these agents may either be competitive or noncompetitive, depending on the inhibitor and on the source of the enzyme. These compounds, like substrate analogs, have been used to inhibit PYRase activity, e.g., they have been used to study the involvement of PYRase in hormone degradation.[116,122,123]

Concerning the effects on PYRase activity as a function of enzyme origin, Prasad[124] has demonstrated that for rat PYRase, activity could either be activated or inactivated depending on the tissue from which the enzyme was isolated. This observation suggested that there were at least two types of PYRase in rat, one inhibitable and the other stimulated by disulfide bond-reducing agents. At least two PYRases with different activities have also been shown to occur in humans.[4,14,98,102]

We conclude this section by re-emphasizing the existence of at least two classes of PYRases: one including the bacterial and animal type I PYRases and the other including the animal type II and serum PYRases. The first class appears to have a broad substrate specificity; most polypeptides ending with an N-terminal pGlu are recognized. The second enzyme class, on the other hand, appears to be highly specific for the pGlu-His bond, as evidenced by the high specificity for TRH. Furthermore, the enzymes of these two classes are not activated or inhibited in the same way. It is noteworthy that a particular PYRase has been reported in rat submaxillary gland by Bharadwaj et al.[125] These authors suggested on comparison of its properties that it may be different from other reported PYRases. However, since it was not purified, it will be difficult to state that this enzyme was really different from others. Phylogeny studies based on these different classes of PYRases would be of great interest.

STUDIES OF PYRase GENES, EXPRESSION, AND PRIMARY STRUCTURE

Although many biochemical and enzymatic studies have been carried out on PYRases from different organisms (Table II), genetic determinants are just now beginning to be characterized. We initiated the cloning of PYRase genes, from two gram-positive bacteria, *S. pyogenes*[104] and *B. subtilis*,[105] and from a gram-negative bacterium, *P. fluorescens*.[93] We named these genes *pcp* because they code for *p*yrrolidone *c*arboxyl *p*eptidase. The strategy we used for isolating these genes was to screen bacterial gene libraries for PYRase activity in *E. coli;* this was possible because this host does not exhibit PYRase activity. Clones were selected using an in situ detection method[104] based on the PYRase assay of Mulczyk and Scewczuk,[126] which uses the chromogenic substrate L-pyroglutamyl-β-naphthylamide. This approach has also been used to isolate the *pcp* gene from *B. amyloliquefaciens*.[106]

The four *pcp* genes characterized so far (all bacterial genes), appear to have a common structure. The size of their open reading frame (ORF) is similar and relatively small. The *pcp* genes from *S. pyogenes, B. subtilis,* and *B. amyloliquefaciens* are all 645 nucleotides long, and the gene from *P. fluorescens* is 639 nucleotides long. These genes encode polypeptides of 215 or 213 amino acids with deduced MWs of 23,135, 23,777, 23,286, and 22,441, respectively. The ORF of the four genes was proceeded from putative ribosome binding sites that ressemble those of *E. coli.* Similarly, the −35 and −10 regions of the putative promoters identified are nearly identical to respective consensus sequences in *E. coli,* and they have the 17 nucleotide spacing that is typical of strong transcriptional signals in *E. coli*.[127] The presence of multiple putative RNA polymerase binding sites was observed in the *pcp* gene from *S. pyogenes,* but only one was characterized as being functional in *E. coli* by primer extension experiments.[104] The presence of inverted repeats upstream the ORFs of the

pcp genes from *B. subtilis* and *S. pyogenes,* which occur within the promoter region in the latter, suggests that these genes may have regulatory binding sites that would be of great interest to investigate.

Inverted repeats have also been identified downstream from the termination codon of the *pcp* genes from *S. pyogenes, B. subtilis,* and *B. amyloliquefaciens.* The stem-loop structures that these repeats may form are similar to the *rho*-independent terminator sequence in *E. coli.*[128] Free energy calculations of the stem-loop structures of the *S. pyogenes* and *B. subtilis pcp* genes, together with the mapping of the 3′ end of their respective transcripts, suggest that no additional protein is required for *pcp* transcription termination; no termination signal, however, was observed for *P. fluorescens* gene.[93]

The *pcp* gene seems to be present as a single copy gene in the bacterial genome. This was confirmed for *S. pyogenes, B. subtilis,* and *P. fluorescens.*[93,104,105] In the case of *S. pyogenes,* Northern experiments have shown that its *pcp* mRNA is monocistronic and that this gene does not belong to an operon.[104]

The GC content of *pcp* genes from gram-positive bacteria is significantly higher than that observed in the genome of these respective bacteria. The GC contents of these *pcp* genes are 43%, 50%, and 55% for *S. pyogenes, B. subtilis,* and *B. amyloliquefaciens,* respectively, while the average GC content for group A streptococci is 37%,[129] for *B. subtilis* 43%,[130] and for *B. amyloliquefaciens* 45%.[130] In contrast, the GC content of the *pcp* gene from *P. fluorescens* (60.7%) is equal to the GC content of the genome of this gram-negative bacterium (60%).[130] These observations have led us to suggest that the *pcp* genes of gram-positive organisms may have been acquired from gram-negative organisms, or at least from a bacterium from which these genes have not yet evolved to conform to the GC content of their respective hosts. This hypothesis is strengthened by the fact that PYRase activity has been reported to be associated with gram-negative bacteria other than *P. fluorescens,* such as *Citrobacter freundii, Enterobacter aerogenes,* and *Yersinia enterolitica.*[131]

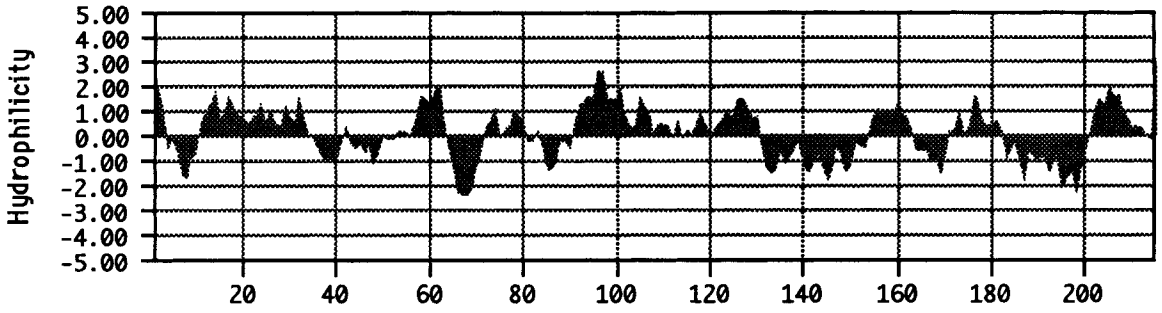
The question of PYRase expression has been approach from several angles. The analysis of *pcp* transcripts by Northern experiments has shown that *pcp* mRNA is present at a very low copy number in *S. pyogenes* and *B. subtilis.* This finding suggests that either the stability of this RNA is low or that the *pcp* gene is weakly expressed, due to an unknown transcription control mechanism, which is consistent with the presence of inverted repeats upstream of the ORFs in the *S. pyogenes, B. subtilis,* and *P. fluorescens* genes. At the protein level, calculations of optimal codon usage for *pcp* genes sequences, according to the list of codon preferences of tRNA in *E. coli,*[132] yield values indicative of low-level expressed proteins. Since the rule of codon usage may be extended to non-*E. coli* microorganisms,[133] we might expect that *pcp* gene expression in the natural host bacteria would also be low. Low-level expression of PYRase has been shown in *S. pyogenes* and *B. subtilis* by Western blot analysis.[92] On the other hand, the overexpression of *pcp* genes from *S. pyogenes* and *B. subtilis* has been successfully performed in *E. coli* using the pT7 system,[134] with expression being greater in the former than in the latter. Likewise, the poor expression of the *pcp* gene from *P. fluorescens* in *E. coli* might be due to protein instability resulting from the relatively high hydrophobicity of the *P. fluorescens* enzyme (see below).

The hydrophobic character of the four PYRases, as determined by the rules of Kyte and Doolittle,[135] indicates that the charge of these peptidases is uniformly distributed along the polypeptide chain (Fig. 3). This is consistent with the observation that these enzymes are soluble. This comparison of hydrophilicity indicated that *B. subtilis* and *B. amyloliquefaciens* are highly related and are more hydrophilic than the PYRases from *S. pyogenes* and *P. fluorescens,* the latter being the most hydrophobic. However, with respect to the elution characteristics during the purification process on hydrophobic interaction columns,[91,92] the hydrophobicity of *B. subtilis* PYRase appeared to be greater than that of *S. pyogenes,* suggesting that folding in the case of *B. subtilis* protein may result in a higher exposure of hydrophobic residues.

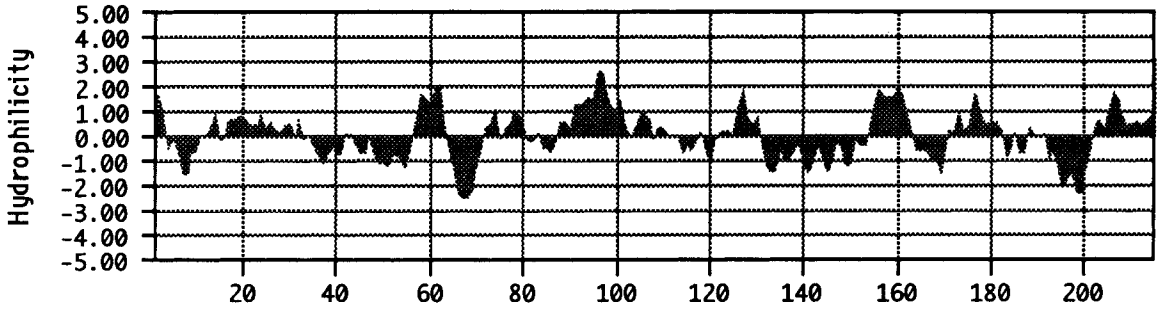
None of the nucleotide sequences present in Genbank release no. 81 (March, 1994) showed significant similarity to the *pcp* genes. Other computer-assisted searches (EMBL release no. 37, SWISS-PROT release no. 27) also failed to reveal any significant amino acid homology between PYRases and other protein sequences from either prokaryotic or eukaryotic sources. This lack of homology to other proteins, including other proteases, indicated that the PYRases belong to a new class of peptidases. On the other hand, the comparison of the deduced amino acid sequences of the four enzymes reported so far pointed out striking similarities (Fig. 4A). This suggests that these genes encoding PYRases may derive from a common ancestor, as suspected above from GC content analysis.

The alignment of PYRase sequences (Fig. 4A) revealed that the primary structure of these proteins is very conserved. The least amount of identity between these enzymes is 31% and the greatest is 72% (Fig. 4B). Two domains of 20 amino acids are located in the central part of these polypeptides, ^{78}ERVAINXX(D)ARIPDN(E)GXOP^{97} and ^{127}G(IP)AXVSXTAGTFVCNX(LF)Y^{146} (*S. pyogenes* protein numbering; residues between parentheses correspond to amino acids present at this site at a frequency equal to 75%); these domains exhibit a large degree of conservation among the four bacterial PYRases. The identity within these domains is 75%

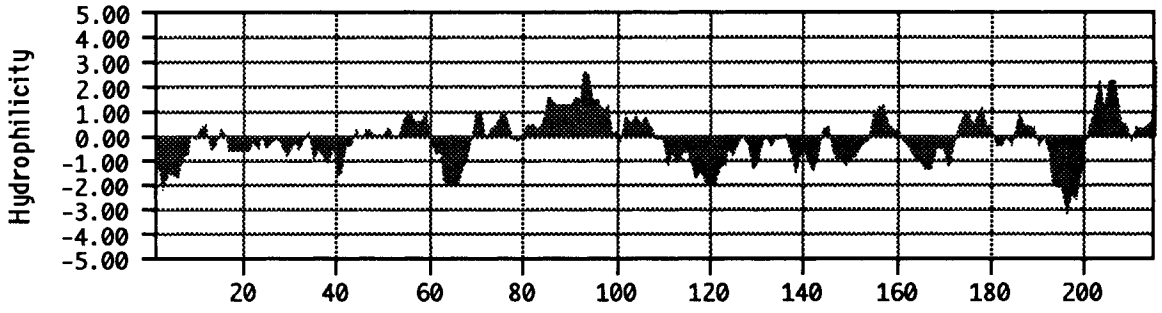
B. subtilis



B. amyloliquefaciens



S. pyogenes



P. fluorescens

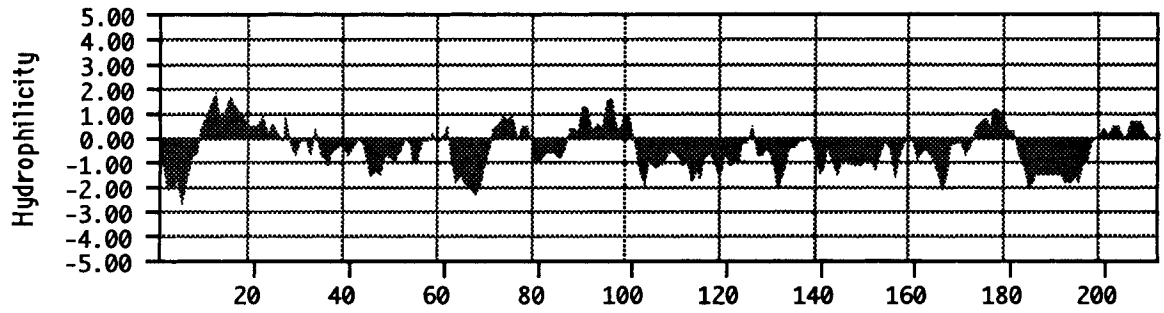


Fig. 3. Hydrophilicity profiles from PYRases. Analysis was performed using a window size of seven residues, according to the rules of Kyte and Doolittle,[135] using the MacVector software (International Biotechnologies, Inc., New Haven, CT).

A

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P. fluo      M-RIVLLTGF EPFDQDPVNP SWEAVRQLDG VQLGSDVKIV ARRIPCAFAT   49
B. sub       MRKKVLLTGF DPFDKETVNP SWEAAKRLNG FETE-EAIIT AEQIPTVFRS   49
B. amy       MEKKVLLTGF DPFGGETVNP SWEAVKRLNG AAEG-PASIV SEQVPTVFYK   49
S. pyo       M--KILVTGF DPFGGEAINP AIEAIKKLPA TIHG--AEIK CIEVPTVFQK   46
Consensus    M..KVL.TGF DPF..E.VNP SWEA.K.L.G ...G-.A.I. ...PTVF..   50

P. fluo      AGECLTRLID ELHPAMVIAT GLGFGRSDIS VERVAININD ARIPDNLGEQ   99
B. sub       ALDTLRQAIQ KHQPDIVICV GQAGGRMQIT PERVAINLAD ARIPDNEGHQ   99
B. amy       SLAVLREAIK KHQPDIIICV GQAGGRMQIT PERVAINLNE ARIPDNEGNQ   99
S. pyo       SADVLQQHIE SFQPDAVLCI GQAGGRTGLT PERVAINQDD ARIPDNEGNQ   96
Consensus    ...VL...I. ..QPD.VIC. GQAGGR..IT PERVAIN..D ARIPDNEG.Q  100

P. fluo      PIDTAVVADG PAAFFTTLPI KAMVKAVREA GIAASVSQTA GTFVCNQVFY   149
B. sub       PIDEEISPDG PAAYWTPLPV KRMTAKMKEH GIPAAVSMTA GTFVCNYLFY   149
B. amy       PVGEDISQGG PAAYWTQLPI KRIVEEIKKE GIPAAVSMTA GTFVCNHLFY   149
S. pyo       PIDTPIRADG KAAYFSTLPI KAMVAAIHQA GLPASVSNTA GTFVCNHLMY   146
Consensus    PID..I..DG PAAY.T.LPI K.MV..... GIPA.VS.TA GTFVCN.LFY   150

P. fluo      LLQHALA--G SGVRSGFIHV PFLPEQVAGS -QRPSMALDA MVAGLQAAVL   196
B. sub       GLMDHISRTS PHIRGGFIHI PYIPQQTIDK -TAPSLSLDT IVRALRIAAV   198
B. amy       GLMDEISRHH PHIRGGFIHI PYIPEQTLQK -SAPSLSLDH ITKALKIAAV   198
S. pyo       QALYLVDKYC PNAKAGFMHI PFMMEQVVDK PNTAAMNLDD ITRGIEAAIF   196
Consensus    .L........ P..R.GFIHI P..PEQ...K -..PS..LD. I...L..A..   200

P. fluo      TAWHTPV--D VKEAGGQVS                                     213
B. sub       TAAQYDE--D VKSPGGTLH                                     215
B. amy       TAAVHED--D IETGGGELH                                     215
S. pyo       AIVDFKDRSD LKRVGGATH                                     215
Consensus    TA.....--D .K..GG..H                                     219
```

B

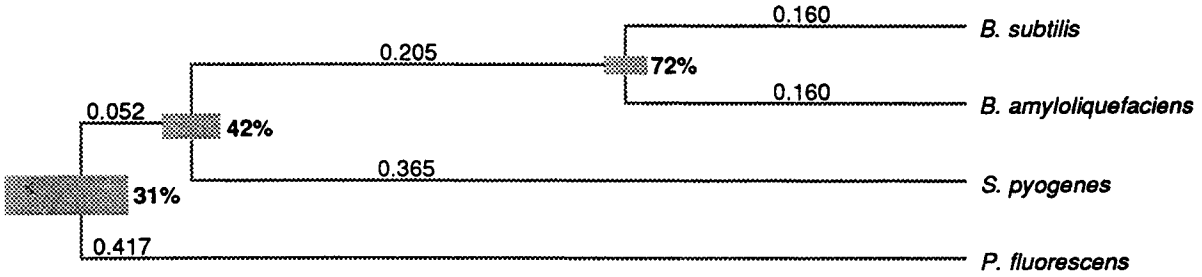

Fig. 4. Alignment and phylogenetic relationship of bacterial PYRases. **A:** Optimal alignment of amino acid sequences from the four PYRases and consensus definition. Identities within the four proteins are boxed. The consensus showed corresponds to the amino acid sequence in which each residue occurs at 75% at that site of the different forms of PYRases. **B:** Tree showing the calculated evolutionary relationship of sequences aligned in A. The length of horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. Scores on each line reflect the phylogenetic relationship of one sequence to another or to a consensus (branch point), values being inversely proportional to the relationship. The percentage at each branch point indicates the identity of sequences coming from this point. The method used for the generation of the phylogenetic tree was the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), based on the simple minimal distance clustering algorithm.[191] Analyses were performed using the GeneWorks software (Intelligenetics, Inc., CA). P. fluo, *Pseudomonas fluorescens;* B. sub, *Bacillus subtilis;* B. amy, *Bacillus amyloliquefaciens;* S. pyo, *Streptococcus pyogenes.*

and 65%, respectively, and on the basis of the 75% consensus definition, these identities increase to 85%. Based on this conservation, these two domains are likely to be involved in the biological activity of PYRases. The second domain that contains a cysteine residue (C^{141}, underlined bold type) appears to be functionally important and may form part of the catalytic site of these enzymes. As discussed above, biochemical studies[8,90] indicate that sulphydryl groups are essential for PYRase activity and that this enzyme belongs to the group of SH-enzymes. Although four cysteine residues are present in the *S. pyogenes* PYRase, only two cysteines (C^{65} and C^{141}) appear to be conserved in the *S. pyogenes, B. subtilis,* and *B. amyloliquefaciens* PYRases (Fig. 4A). Sequence alignment revealed, however, that C^{65} in the *P. fluorescens* enzyme was not a conserved residue and that C^{141} was therefore probably responsible for biological activity. This analysis has been confirmed by site-directed mutagenesis of the *B. amyloliquefaciens* PYRase.[106] Substitution of the C^{65} with serine did not affect the enzyme activity, whereas substitution of C^{141} resulted in a complete loss of enzyme activity. Thus, the second conserved domain (represented by residues 127–146 in the *S. pyogenes* numbering; Fig. 4A) appears to be required for catalytic activity. Moreover, depending on protein folding or the rearrangement of subunits (some PYRases are multimers, as discussed above), this second domain might partake in catalytic activity.

A phylogenetic analysis of the bacterial PYRases characterized so far (Fig. 4B) indicates that the *B. subtilis* and *B. amyloliquefaciens* enzymes are closely related (72% identity). The location of the branch point of these two enzymes on the phylogenetic tree suggests that they may belong to a separate PYRase class, compared with the *S. pyogenes* and *P. fluorescens* enzymes. This relatedness is consistent with the fact that *B. subtilis* and *B. amyloliquefaciens* are highly related species belonging to the same genus.[130] Surprisingly, the enzyme from *S. pyogenes* appears to be more highly related to the *P. fluorescens* enzyme than the *Bacillus* enzymes, although these organism are, like *S. pyogenes,* gram positive. The location of the *P. fluorescens* branch point is consistent with our hypothesis that, from an evolutionary point of view, the PYRase genes may have been derived from a common ancestor belonging to the gram-negative bacteria. In considering this, it would be of great interest to characterize PYRase genes from other gram-negative bacteria. Finally, since no data on mammalian PYRase genes have been published at this time, we conclude this section by encouraging the cloning and sequencing of PYRases from mammals. This may give more information about the possible function and role of these enzymes in central nervous system.

LOCALIZATION AND PUTATIVE ROLE OF PYRases

The various sources from which PYRases have been isolated are summarized in Table II. Except for the *K. cloacae* enzyme, which is associated with a "particulate" fraction,[8] as is the mammalian type II PYRase, all the bacterial PYRases have been shown to be soluble proteins. Although not proved in all cases, these enzymes appear to be located in the cytosol of bacterial cells. Tsuru et al.[90] have shown that the PYRases of *B. amyloliquefaciens* and two strains of *B. subtilis* (DT-39 and ML-208) are cytoplasmic enzymes. Moreover, this enzyme has also been found in the cytoplasm of the bacteria *S. pyogenes*[91,104] and *B. subtilis*.[105] Such solubility and intracellular localizations are consistent with the primary structure of these PYRases, which suggests that these enzymes are not exported from cells and that they are not membrane bound.

Although not yet investigated in enough cases, one might expect that the bacterial PYRases are involved in intracellular protein metabolism. Generally speaking, the bacterial aminopeptidases are thought to be involved in protein maturation, protein degradation, and the utilization of peptides as nutrients.[136] Such a proposed role for the bacterial PYRases is, however, weakened by the observation that these enzymes are not commonly found in bacteria. It is noteworthy that even within the same species, some strains have PYRase activity, while others lack this activity.[126,137,138]

A possible role for PYRases in nutrient metabolism has been suggested in *P. fluorescens,* which was able to grow on free pGlu as the sole source of carbon and nitrogen.[5] After intracellular transport, pGlu could conceivably have been utilized as a nutrient source through its removal from pGlu-terminating peptides by PYRase. However, other aminopeptidases with broad substrate specificity are generally also present and are probably more likely to be involved in nutrient metabolism.[136] Moreover, the substrate specificity of PYRases suggests that they have a more specific role in bacteria. It would be of great interest to investigate further the physiological role of this enzyme in bacteria and, in particular, to answer the question as to why this enzyme is present in some bacterial strains and not others.

PYRase may be involved in detoxification, since in our opinion, the high accumulation of peptides with a pGlu N-terminus may abnormally acidify the cell cytoplasm. In this regard, it would be of interest to determine whether those bacterial strains that lack PYRase activity have 5-oxoprolinase activity, since this enzyme can decyclize pGlu and thus it may play a role similar to that of PYRase in detoxification.

In contrast to the situation observed in bacteria, PYRases seem to be more widely distributed in an-

imals, particularly in mammals. As previously mentioned, at least two types of PYRases are found in animals: type I PYRase, which is a soluble protein, and type II PYRase, which is membrane bound; a third PYRase, serum PYRase, resembles the type II enzyme and has been referred to as thyroliberinase.[2]

The isolation of type I PYRase from the soluble fraction of many mammalian tissues suggests that it is a cytosolic enzyme.[4,96,102,109] This enzyme appears to have a wide tissue distribution: nearly the same content of PYRase I is found in mammalian brain, hypothalamus, pancreas, liver skeletal muscle, and kidney tissues.[100,139]

In the case of type II (membrane-bound) PYRases, their catalytic site appears to be exposed extracellularly.[140–143] Although present in many mammalian tissues, type II PYRase seems to be primarily located in the central nervous system.[100,143,144] In the central nervous system, this enzyme is found associated with the synaptosomal membrane.[110,145,146] The more limited tissue distribution of type II PYRase differs from the generally broad distribution of peptidases that are believed to be involved in the catabolism of select peptides.

As stated above, PYRase II has specificity for TRH and closely related peptides.[147] The TRH tripeptide pGlu-His-ProNH2 stimulates the release of pituitary thyrotropin [thyroid-stimulating hormone (TSH)], prolactin, and growth hormone. It may also act as a neurotransmitter or neuromodulator in the central nervous system.[13,148] Some studies have indicated that this tripeptide can potentiate the behavioral effect of L-dopa and pargyline in rat brain[149] and to increase cerebral noradrenaline turnover.[150] Finally, TRH was shown to be significantly increased in T3- and T4-induced hyperthyroidism and significantly reduced in thiouracil-induced hypothyroidism.[151]

Based on its localization and specificity, PYRase II is thought to be involved in regulating the biological activity of neuronally released TRH. Two pathways have been shown to be involved in the metabolism of TRH. In the deamidase pathway, the Pro-NH2 bond is cleaved first by a prolyl endopeptidase (E.C. 3.4.21.26).[100] In the second pathway, the pGlu-His bond is first cleaved by PYRase II.[152–154] Direct and indirect evidence support the involvement of PYRase II in the extracellular breakdown of TRH in brain tissue. For example, the inhibition of type II PYRase activity has been shown to increase the recovery of TRH from brain tissue.[155] It has also been suggested that serum PYRase, which may be the secreted form of liver PYRase II, may also be involved in TRH catabolism.[14,101] Rat and human sera forms of PYRase II appear to be different with respect to TRH degradation: in rat there is a marked effect of the state of the thyroid on TRH degradation in serum, whereas in human there is no significant effect.[156,157] Another observation that supports PYRase II involvement in TRH metabolism is that its activity appears to be regulated by estrogen and thyroid hormone.[13,158] Thus, it has been proposed that the degradation of TRH by PYRase II may play a role in the negative feedback control of PYRase activity; feedback control by thyroid hormones involves multiple levels of regulation including the inactivation of TRH.[158]

PYRase II catalysis of TRH in the central nervous system yields His-Pro-NH2, which can cyclize to give His-Pro-diketopiperazin (His-Pro-DKP).[159,160] TRH and His-Pro-DKP have been reported to play important roles in mammalian physiology by influencing central nervous system and adenohypophysial functions.[161] With regard to the involvement of PYRase II, it appears to play as important a physiological role as other neuropeptide-degrading enzymes.

PYRase I, the soluble PYRase, has been shown to act on many different substrates having an N-termainal pGlu. In fact, this PYRase has been shown to hydrolyze the N-terminal pGlu group of neuropeptides, including TRH. This PYRase, however, does not appear to be involved in the control of TRH levels, as is type II PYRase.[102,139,141,162,163]

The presence of two enzymes acting similarly in the same system suggests that the enzymes may not necessarily be involved in the regulation of the same physiological pathway. If the role of type II PYRase has been identified, the role of type I PYRase remains unclear. It has been proposed,[102,139] through comparison to other soluble aminopeptidases, that type I PYRase may contribute to the final stages of the intracellular catabolism of peptides to free amino acids, which are then released to the cellular pool. Thus, this enzyme may, at least in part, be involved in the regulation of the cellular pool of free pGlu. It is noteworthy that free pGlu is known to have pharmacological properties (see above); thus a specific pathway for pGlu production, e.g., through PYRase I activity, may exist to generate this molecule. The source of the release of free pGlu that is associated with certain diseases remains unknown, but the involvement of PYRase remains a possibility.

APPLICATIONS INVOLVING PYRase

The discovery of PYRase was initially associated with the discovery of an enzyme that could remove the N-terminal pGlu block to terminal amino acid sequencing by Edman[164] sequential degradation.[5,9,165] Nowadays, even though enzymatic[84] and chemical methods[28,166,167] are available to open pyrrolidone rings, and physical methods, such as mass spectrometry, are available to overcome sequencing difficulties due to the N-terminal pGlu block,[30,168,169] PYRase is still used by sequencers to confirm the presence of this residue.[168–170]

The importance of PYRase has been elevated by

its use in bacterial diagnosis. In the 1980s, detection methods were developed in bacterial diagnosis based on the use of chromogenic and fluorogenic substrates.[138,171–186] Because PYRase has a specific enzymatic activity, chromogenic and fluorogenic substrates have been developed to differentiate bacteria based on PYRase activity. Such substrates included: L-pyroglutamyl-β-naphthylamide,[6] L-pyroglutamyl-*p*-nitroanilide, and L-pyroglutamyl-7-amido-4-methylcoumarin.[112] Initial applications of the "PLP" test, developed by Mulczyk and Szuwczuk,[126,137] included differentiation of the enterobacteriacea and staphylococci species. Godsey et al.[171] later described the hydrolysis of L-pyroglutamyl-β-naphthylamide by PYRase in the identification of group A streptococci and enterococci. Bosley et al.[173] showed that *Enterococcus faecalis* and *Streptococcus bovis* biotype I could be identified in a 4-hour assay. Oberhofer[178] has described a "PYR" test to identify group A streptococci and enterococci, as well as *Streptococcus haemolyticus* and the staphylococci. Short methods, requiring only minutes, have also been developed based on the detection of PYRase activity. Ellner et al.[175] reported a rapid colorimetric test based on PYRase in the identification of group A streptococci and enterococci. Rapid "paper strip" tests have been described by Wassilauskas and Hampton[174] (Strep-A-Fluor paper strip test, Bio Spec, Dublin, CA), Oberhofer[178] (Minitek PYR disks, BBL Microbiology Systems, Lokeyville, MD; Strep-A-Chek, EY Laboratories, San Mateo, CA), Kaufhold et al.,[182] and Dealler et al.[186] (Strep Strip, Lab M). The identification of bacteria based on PYRase activity can also be performed in liquid culture and on agar media.[187] In addition, tests have been developed for direct bacterial identification from clinical human and animal samples.[181,182,188] It is worth noting that the PYRase test alone may not be sufficient for bacterial (pathogen) identification when applied to clinical samples, and thus this test is usually accompanied by other enzymatic tests and serological tests for confirmation.

Beyond the detection of PYRase activity in bacterial diagnosis, the characterization of PYRase genes could also lead to promising applications.[104] For example, group A streptococci have been identified using DNA probes to the PYRase gene.[189] With this approach, it may be possible to replace the PYRase activity test with a molecular probe test for a more definitive identification of bacterial species. Finally, since PYRase activity can be detected in situ, and can be assayed for in vivo and in vitro using chromgenic substrates, it has been proposed that the PYRase gene could be used as a reporter gene.[104]

Activators and inhibitors of PYRases would be valuable tools to probe the function of these proteins and could prove to be valuable therapeutic agents. For example, the protection of the pyroglutamyl residue of peptides from PYRase attack may improve the delivery of these peptides in therapeutics.[190] N-L-pyroglutamate derivatives of various anticancer drugs have been proposed as potential "prodrugs." Such derivatives are designed to be cleaved to the active cytotoxic agents by the PYRase at the tumor site. For targeting the tumor site, a PYRase chemically linked to a monoclonal antibody may be useful (Dr. Cheung, personal communication).[192]

CONCLUSIONS

PYRase is an enzyme widely distributed among plants, animals, and bacteria. These enzymes appear to play an important role in the activation and inactivation of many N-terminal pGlu-terminating peptides. In bacteria, PYRases first became of interest because of their application to N-terminal pGlu unblocking prior to Edman sequential degradation in peptide and protein sequencing. They were initially discovered in bacteria and their activity has now proved useful in bacterial diagnosis. In animals, especially in mammals, this enzyme has been shown to be principally involved in neuropeptide metabolism.

Biochemical and enzymatic characterization of the PYRases has revealed many differences among the bacterial and animal enzymes. Only one type of PYRase has been found in bacteria, whereas two types (type I and type II) have been characterized in mammals. Though similar in many respects, such as in their broad substrate specificity and other physico-chemical properties, bacterial PYRases are generally oligomers, but the animal type I enzyme appears to be monomeric. Type II and serum PYRases in animals are enzymatically and structurally different from type I and bacterial PYRases. Although PYRases have been widely studied from biochemical and enzymatic standpoints, characterization of their genes and structures is only beginning. To our knowledge, only four bacterial PYRases genes have been characterized. Their deduced amino acid sequences reveal striking similarities and suggest relatedness. The molecular characterization of more genes in this peptidase class, especially the animal type I and type II enzymes, would lead to a better understanding of these enzymes. Sequence comparisons have already identified the plausible catalytic site and show possible phylogenic relationships. Moreover, since large amounts of PYRase can be produced by overexpression, crystallization of this enzyme and a study of three-dimensional structure is imminent. It is worth noting that crystallization of the *B. amyloliquefaciens* PYRase has already been achieved, and such an investigation is also in progress in our laboratory.

Although a possible physiological role exists for the animal type II PYRase, its role in bacterial protein metabolism remains hypothetical. This enzyme may have another function in bacteria, e.g., in nutrient metabolism. Further investigations are re-

quired to improve our understanding of the role of this enzyme in cell and tissue metabolism.

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