Stability and Kinetic Studies on Recombinant Human Pyroglutamyl Peptidase I and a Single-Site Variant, Y147F

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Abstract

Human brain pyroglutamyl peptidase (PAPI; EC 3.4.19.3) is an omega exopeptidase which cleaves pyroglutamic acid from the N-terminus of bioactive peptides and proteins. It plays an important role in the processing and degradation of regulatory peptides such as thyrotropin releasing hormone (TRH) and luteinizing hormone releasing hormone (LHRH). To gain further insights into its performance *in vivo* and suggest possible applications, such as peptide processing or sequencing, this study focuses on the *in vitro* stability properties and Michaelis-Menten kinetics of the recombinant wild type enzyme and a single-site mutant, Tyr147→Phe (Y147F).

At 60ºC in 50mM potassium phosphate buffer, pH 8.0, recombinant PAPI underwent a first-order decay constant with a *k* value of 0.046 ± 0.002 min^{-1} and a half-life of 15 min. PAPI was unstable to most of the water-miscible solvents tested (dimethyl sulphoxide, methanol, acetone, tetrahydrofuran, acetonitrile, dimethyl formamide and ethanol) even at low v/v concentrations. Methanol and dimethyl sulphoxide were the least injurious to PAPI activity: 56% and 50% residual PAPI activity remained at 10% v/v methanol and DMSO, respectively. Chemical modification with dimethyl suberimidate gave only 20% recovery of initial activity and did not stabilize the enzyme. Polyol and other stabilizing additives were investigated: activity and stability increased with xylitol but not with trehalose, glycerol or ammonium sulphate. PAPI displayed Michaelis-Menten kinetics with the fluorescent substrate pyroglutamyl 7-aminomethylcoumarin at pH 8.0: values for K_m and k_{cat} were 0.132 ± 0.024 mM and 2.68 ± 0.11 × 10^{-5} s^{-1} respectively.

Mutant Y147F was notably more thermostable, despite differing from wild type only by the absence of a hydroxyl. At 70ºC, the Y147F first-order *k* value was 0.0028 ± 0.001 min^{-1} (half-life 25 min) compared with 0.079 ± 0.003 min^{-1} (half life 9 min) for wild type (the higher temperature was required to achieve timely inactivation of Y147F). Values of K_m and k_{cat} for Y147F (0.115 ± 0.019 mM and 2.45 ± 0.05 × 10^{-5} s^{-1} respectively) closely resembled those of wild type.

It appears that the *in vitro* stability of wild type PAPI might limit its potential applicability in peptide processing or other fields. Additives and chemical modification seem to have limited scope for enhancing its stability but the generation of stabilized mutant variants, or the use of a thermophilic counterpart, should be explored further.

Abbreviations
ACN, acetonitrile; ALT, Alanine aminotransferase; AMC, 7-Amino-4-methyl-coumarin; BCA, Bicinchoninic acid; BSA, Bovine serum albumin; C_{50}, Solvent concentration where 50% of enzyme activity in aqueous buffer remains; DMF, Dimethylformamide; DMSO, Dimethylsulphoxide; DMS, Dimethyl suberimidate; DTT, Dithiothreitol; EDAC, *N*-(3-Dimethylaminopropyl)-N'-ethyl carbodiimide; EDTA, Ethylene diamine tetra-acetic acid; F16Y, PAPI mutant Phe16→Tyr; GRH, Gonadotropin releasing hormone; HRP, Horseradish peroxidase; HPLC, High performance liquid chromatography; IPTG, Isopropyl-beta-D-thiogalactopyranoside; *k*, First-order decay constant; LB, Luria-Bertani medium; LHRH, Luteinizing hormone-releasing hormone; MeOH, Methanol; MEROPS, the peptidase database at http://merops.sanger.ac.uk/; N, Native state of protein; β-NA, β-Naphthylamine; NEM, N-Ethylmaleimide; PAPI , Pyroglutamyl peptidase I (E.C.3.4.19.3); PAPII, Pyroglutamyl peptidase II (E.C.3.4.19.6); pGDMK, pGlu diazomethyl ketone; pGCK, pGlu chloromethyl ketone; pGlu, Pyroglutamic acid; pGlu-AMC, Pyroglutamyl-7-amino-4-methyl coumarin; pNa, p-Nitroanilide; RIA, Radioimmunoassay; SDS, sodium dedecyl sulfate; T_{50}, Half-inactivation temperature; $t_{½}$, Half-life (0.693/*k*); THF, Tetrahydrofuran; TRH, Thyrotropin releasing hormone; Y147F, PAPI mutant Tyr147→Phe; Z, Benzyloxycarbonyl.

Introduction
Pyroglutamyl peptides, which often comprise up to 40 amino acids, possess an N-terminal pyroglutamic acid (pGlu) residue that influences their biological properties. Many reports describe the enzymatic formation of pGlu from glutamic acid and glutaminyl peptides (Orlowski and Meister 1971). Cyclisation of the N-terminal glutamic acid to pGlu affords these peptides a longer half-life than others of similar size (De Gandarias *et al*, 2000). Many biologically active peptides (thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), neurotensin, etc.) and proteins have pGlu residues at their N-termini. Only the pyroglutamyl aminopeptidases can cleave these amino terminal pGlu. Three types of pyroglutamyl peptidase have been found in a wide variety of bacteria and in many plant, animal, and human tissues. These are pyroglutamyl peptidase I (E.C 3.4.19.3; PAP I), pyroglutamyl peptidase II (E.C 3.4.19.6; PAP II) and serum thyroliberinase (E.C 3.4.19.6) (Cummins and O'Connor, 1998; Robert-Baudouy and Thierry, 1998). In the brain, the cytoplasmic cysteine peptidase PAP I (EC 3.4.19.3, the subject of this paper) hydrolyses pGlu-X bonds (where X is any amino acid except proline) and is quite distinct from the membrane-bound, tetrameric, exo-acting metallo peptidase PAP type II (EC 3.4.19.6) that degrades thyrotropin releasing hormone in a highly specific manner (O'Connor and O'Cuinn, 1985).

Classification and occurrence of PAPI
Pyroglutamyl peptidase I (PAPI, EC 3.4.19.3) is a cysteine omega-exopeptidase that specifically removes the amino-terminal pyroglutamyl residue from oligopeptides and proteins. It has been used in protein sequencing to unblock proteins and polypeptides with pGlu amino-termini prior to Edman degradation and has had several different names, including pyrrolidonyl peptidase, pyrrolidone carboxyl peptidase, 5-oxoprolyl-peptidase, pyrase and pyroglutamyl aminopeptidase (Cummins and O'Connor, 1998).

PAPI has been found in various plant, animal, and human tissues, but none has yet been found in the *Saccharomyces cerevisiae* genome nor in any fungus. It occurs as a soluble, intracellular cytosolic cysteine peptidase with broad specificity for pGlu-substrates in many mammalian tissues including human cerebral cortex, kidney and skeletal muscle, rat, bovine and guinea pig brain, and in various rat organs including liver (Cummins and O'Connor, 1998). In vertebrates, the liver and kidney show relatively high PAPI activities compared with other tissues. PAPI was localized in the renal proximal tubules, while an immunohistochemical localization study of PAPI

demonstrated intracellular distribution in the pituitary. Known mammalian PAPI sequences include those from human (*Homo sapiens*; Dando et al., 2003) mouse (*Mus musculus*; Dando et al., 2003) and rat (*Rattus norvegicus*; Abe et al., 2003).

PAPI activity has also been noted in non-mammalian sources such as bird, fish and amphibian tissues. Among plants, it occurs in parsley, carrot, bean, oats, wheat, cauliflower, and potato. Tissues tested included leaves, seeds, sprouts and roots (Szewczuk and Kwiatkowska, 1970). The purification and study of PAPI has been reported from several prokaryotic species, including species of *Bacillus* (Yoshimoto *et al*., 1993; Awadé *et al*., 1992a) *Pseudomonas fluorescens* (Gonzalès and Robert-Baudouy, 1994), species of *Pyrococcus* (Sokabe *et al*., 2002; Tsunasawa *et al*., 1998), *Streptococcus pyogenes* (Awadé *et al*., 1992b; Cleuziat *et al*., 1992), *Mycobacterium bovis* (Kim *et al*., 2001), *Staphylococcus aureus* (Patti *et al*., 1995), and *Thermococcus litoralis* (Singleton *et al*., 2000). The sequences of bacterial and archaeal PAPI enzymes place them in peptidase family C15 of the MEROPS classification (Barrett and Rawlings, 2001).

Substrates, catalysis and inhibitors

PAPI hydrolytically removes L-pGlu from L-pGlu-L-X, where X is any amino acid (except proline), a peptide or an arylamide such as AMC. PAPI has a broad pyroglutamyl substrate specificity, with its activity being influenced by the amino acid directly after the pGlu residue. Synthetic compounds and dipeptides such as pGlu-AMC, pGlu-Ala, pGlu-pNa, pGlu-βNA and pGlu-Val are also substrates for the enzyme but pGlu-Pro bonds are normally not hydrolysed by mammalian PAPI (Cummins & O'Connor, 1996; Browne & O'Cuinn, 1983). Abe et al. (2004a,b) have shown that PAPI can tolerate some single atom substitutions on the pGlu ring. Due to the catalytic importance of its cysteine thiol group, PAPI has an absolute requirement for a thiol-reducing agent such as DTT and loses activity when treated with micromolar concentrations of a standard thiol inhibitor such as N-ethylmaleimide (NEM) or iodoacetate (Cummins & O'Connor, 1996; Tsunasawa et al, 1998; Singleton et al, 2000; Singleton & Littlechild, 2001; Dando et al, 2003). The additional involvement of a histidine and of acidic residues in catalysis was indicated by chemical modification studies with diethylpyrocarbonate and N-(3-Dimethylaminopropyl)-N'-ethyl carbodiimide (EDAC) respectively (Le Saux et al, 1996). Several specific inhibitors have been synthesized to elucidate the biological significance of the enzyme (Fujiwara et al, 1981; Friedman et al, 1985), including pGlu chloromethyl ketone (pGCK), Z-pGlu chloromethyl ketone (Z-pGCK), and Z-pGlu diazomethyl ketone (Z-pGDMK). Charli et al. (1987) reported that pGDMK did not enhance brain TRH levels in vivo or in vitro, but Faivre-Bauman et al. (1986) observed increased levels of TRH when primary hypothalamic cell cultures were treated with Z-Gly-Pro-CHN_2, another PAPI inhibitor. Fujiwara et al. (1981) noted a decrease in enzyme activity upon addition of pGCK in the absence of DTT. A synthetic aldehyde analog of pGlu, 5-oxoprolinal, was a potent competitive inhibitor of PAPI (Friedman et al, 1985). Several other inhibitors of mammalian PAPI exist. Yamada and Mori (1990) noted the inhibitory effects of 1,10-phenanthroline, excess DTT and EDTA while Cummins and O'Connor (1996) reported 28% inhibition of bovine brain PAPI by 1mM 1,10-phenanthroline. Two compounds isolated from the genus *Streptomyces*, benarthin and pyrizinostatin, acted as inhibitors of PAPI (Aoyagi et al, 1992a,b) as did an oligosaccharide gum from *Hakea gibbosa* (Alur et al, 2001). Mantle et al. (1991) noted inhibitory effects of amastatin, arphamenine, chymostatin, elastinal and leupeptin while benzamidine inhibited the bacterial PAPI enzyme (Awadé et al, 1994). Several workers have used the selective reversible inhibitor 2-pyrrolidone to stabilize type I pyroglutamyl peptidases during purification and storage (Mudge & Fellows, 1973).

Characterization
The molecular weight of PAPI monomer has mostly been reported as around 24 kDa by gel filtration and denaturing gel electrophoresis. Optimal activity lies in the pH range 6.0 to 9.5 and, where reported, the isoelectric point (pI) is around 5.0. Optimum temperatures for activity of mesophilic prokaryotic PAPI have mostly been reported as ranging from 30 - 45ºC (37ºC has been widely used as the standard reaction temperature for eukaryotic PAPI) but two thermophilic enzymes from *Thermococcus litoralis* PAPI and *Pyrococcus furiosus* PAPI exhibit optimum activity at 70ºC and 90ºC, respectively. (Singleton & Littlechild, 2001; Ogasahara *et al*, 2001). Purification of PAPI from several strains of *Bacillus* has been well documented. *Bacillus amyloliquefaciens* PAPI has been particularly well studied, including cloning, sequencing and expression of its gene in *Escherichia coli*. The enzyme comprises 215 amino acid residues, has a homodimer structure with a deduced subunit molecular mass of 23.3kDa and a pH optimum of 6.5. (Yoshimoto *et al*, 1993; Ito et al., 2001).

Physiological role(s)
The physiological role of PAPI currently remains unclear. Its cytosolic location excludes a significant role in extracellular peptide degradation (Charli et al., 1987; Abe et al., 2004a,b). O'Cuinn et al. (1990) suggested that PAPI may represent a mechanism for returning pGlu terminating neuropeptides, released from damaged or ageing vesicles, back to the cellular amino acid pool. PAPI may participate in the intracellular catabolism of peptides to free amino acids which are then re-incorporated into biosynthetic pathways. Thus, PAPI may function in regulating the cellular pool of free pGlu. In mammals, it may be involved in the inactivation of biologically active peptides with an N-terminal pGlu, such as TRH, LHRH, bombesin, neurotensin, gastrin, fibrinopeptides and anorexigenic peptide (Alba et al, 1995). PAPI may also contribute to the inactivation of neuropeptides that are produced in excess. Faivre-Bauman *et al*. (1986) showed that addition of specific PAPI inhibitors to cultured TRH-synthesising hypothalamic cells significantly increases TRH content and release from cells under both basal and K^+-stimulated conditions. PAPI is involved in the hydrolysis of some xenobiotic compounds with an L-pGlu or L-pGlu-related structures (Abe *et al*, 2003; 2004b) and may also be involved in detoxification of pGlu-peptides, since high levels of such peptides would abnormally acidify the cell cytoplasm. The high level of Pyroglutamyl Peptidase in the human brain cortex agrees with the observation that inactivation of TRH is higher in the human brain cortex than in other regions (Griffiths, 1985).

Roles in health and disease
A correlation between PAPI activity and TRH levels in mammalian brain has been observed in many studies (De Gandarias *et al*, 1998; 2000). A decrease in PAPI activity coincides with increasing levels of TRH as brain development progresses, indicating that PAPI plays a part in the normal development of mammalian brain. The wide distribution of TRH throughout the central nervous system and the findings of various biochemical, pharmacological and behavioural studies strongly implies that TRH may act as a neuromodulator or neurotransmitter in the extrahypothalamic brain. Also, during earlier stages of development, high PAPI activity is linked to elevated levels of cyclo(His-Pro) (Prasad *et al*, 1983). In addition to its better-known neurohormonal role, PAPI may also have an important part to play in memory and learning, in metabolism and the possible control of conditions such as Alzheimer's disease (Irazusta *et al*, 2002).

The effect of light intensity on the activity of PAPI in the functionally connected rat retina and hypothalamus was studied by Ramírez *et al*. (1991) and Sánchez *et al.* (1996). PAPI levels fluctuate periodically, coincident with environmental light and dark conditions, suggesting a possible function of PAPI within the human "body clock" (Sanchez *et al.* 1996). Substrates of

PAPI (such as TRH or LHRH) may play a functional role in the retina, apart from their well-known role in the hypothalamus. The concentration of TRH in the hippocampus of elderly controls and AD patients was recorded by radioimmunoassay (RIA). He and Barrow (1999) reported that PAPI may be involved in the propensity of amyloid precursors to form insoluble plaques, resulting in Alzheimer-type diseases. Free pGlu is known to have pharmacological properties and these have been demonstrated in certain disease states (Lauffart *et al*., 1989; Mantle *et al*., 1990; 1991).

Active Site

The active sites of cysteine proteases typically have a catalytic triad, an oxyanion hole and a specificity pocket. The catalytic triad of *B. amyloquefaciens* PAPI comprises Cys144, His168 and Glu81 (Odagaki *et al*., 1999), as confirmed by site-directed mutagenesis of the appropriate amino acids. Previously, the two cysteine residues of *B. amyloquefaciens* PAPI, Cys68 and Cys144, had been mutated to Ser. Mutant Cys144→Ser had no detectable PAPI activity (Yoshimoto *et al*., 1993), while Cys68→Ser had wild type activity, implicating Cys144 as the active site thiol. Also, titration with 5,5'-dithio-bis-(2-nitrobenzoate) showed that Cys68 is located internally. His168 is also completely conserved, and thus PAPI was thought to be a cysteine protease with a Cys-His catalytic diad or Cys-His-Asp/Glu catalytic triad.

Le Saux *et al*. (1996) investigated several candidate active site residues of *Pseudomonas fluorescens* PAPI. Substitution of residues Cys144 and His166 by Ala and Ser, respectively, resulted in inactive enzymes. Proteins with changes of Glu-81 to Gln and Asp-94 to Asn were not detectable in crude extract and were probably unstable in bacteria. The results suggest that Cys-144 and His-166 constitute the nucleophilic and imidazole residues of the enzyme active site, while residue Glu-81, Asp-89, or Asp-94 might constitute the third part of the active site (see below). Tsunasawa *et al*. (1998) substituted Cys142 of *P. fluorescens* PAPI with Ser, resulting in inactive enzyme and showed, by sequence analysis, that the catalytic triad Cys142-His166-Glu79 corresponds to Cys144-His168-Glu81 of *B. amyloquefaciens* PAPI. The location of the Cys144 at the N-terminus of an α-helix could be important. Such a position could affect catalysis, as the helix dipole can depolarise the amide bond and enhance its reactivity.

PAPI does not have a well defined oxyanion hole; however, it is possible that a tetrahedral oxyanion could be produced by the contribution of Cys144 and Arg91 and their respective side chains. A hydrophobic region close to Cys144 provides a highly specific binding site for the pGlu of the enzyme's substrate. This pocket appears to have some conformational flexibility, hence allowing for maximum interaction with the substrate. PAPI appears to have only one pocket of specificity (Odagaki *et al*., 1999). The catalytic residues Glu81, Cys144, and His166 of the *B. amyloliquefaciens* enzyme are conserved in the human sequence (Dando et al, 2003).

Structure

Native mammalian PAPI appears to be monomeric, but molecular mass values of 50–91 kDa, consistent with a dimer, have been reported for the bacterial enzyme. The recombinant *B. amyloliquefaciens* enzyme probably functions as a dimer (Yoshimoto *et al*., 1993) but has been crystallised as a tetramer (Odagaki *et al*., 1999) while crystalline PAPI from *Thermococcus litoralis* is also tetrameric (Singleton *et al*., 1999a,b).

The first documented X-ray crystal structures of bacterial PAPI were at 1.6 Å for *Bacillus amyloliquefaciens* (Odagaki *et al*., 1999), at 1.73 Å for *Pyrococcus furiosus* PAPI (Tanaka *et al*., 2001), at 2.0 Å for *Thermococcus litoralis* PAPI (Singleton *et al*., 1999a,b) and at 2.2 Å for *Pyrococcus horikoshii* PAPI (Sokabe *et al*., 2002). The monoclinic crystal form of each one has four crystallographically independent copies of PAPI in the asymmetric unit, and comprises a

tetramer of four identical subunits designated A-D. Each monomer of the tetramer makes contact with two other subunit monomers. The A-D interface of *Bacillus amyloliquefaciens* PAPI involves hydrophobic interactions and several ionic salt bridges between each monomer. The A-C interface does not have any ionic salt bridges and a thin layer of water mediates hydrogen bonds between the subunits (Odagaki *et al*., 1999).

The polypeptide folds in an α/β globular domain with a hydrophobic core comprising a twisted β-sheet surrounded by five α- helices. This structure allows the function of most of the conserved residues in the PAPI family to be identified, and it seems that the catalytic triad comprises Cys144, His168 and Glu 81 (see above) situated inside the doughnut-shaped tetramer.

The *Thermococcus litoralis* PAPI tetramer has a central cavity of 6000 $Å^3$ (Singleton *et al*., 1999a,b). The A-B interface has hydrophobic interactions and salt bridges involving Arg81, Asp88, Asp101 and Arg119. The A-C interface is formed by an extended loop and a disulphide bridge exists between Cys190 of each monomer. This hydrophobic core may contribute towards the thermostability of *Thermococcus litoralis* PAPI. Other residues along this interface are generally hydrophobic.

The A-B interface of the *Pyrococcus horikoshii* PAPI tetramer also involves hydrophobic interactions but there are, in addition, inter-subunit ionic bonds while the A-C interface has hydrogen bonds that are entirely mediated by a thin layer of water; however, the enzyme was a dimer in solution (Sokabe *et al*., 2002).

PAPI Wild Type and Mutant Y147F as Candidates for Study
Recombinant human PAPI may have use in protein sequencing and/or in processing of peptides, and possibly in enzymatic peptide synthesis for the attachment of N-terminal pGlu residues. A thorough understanding of its stability and catalytic properties would be needed for any such applications. This paper reports a study of recombinant human PAPI and its single-site mutant Y147F, previously cloned, characterized and over expressed in *E. coli* (Vaas, 2005), with regard to their stabilities at elevated temperatures and kinetics with the chromogenic substrate pGlu-AMC. In addition, the stability of the wild type recombinant to organic solvents, and the effects on it of additives and of a crosslinking reagent, were also investigated.

Materials and Methods
Fisher Scientific supplied glacial acetic acid, acetone, EDTA, ethanol (100% v/v), glycerol, HCl (37% v/v), NaCl, NaOH, Tris-(hydroxymethyl) aminomethane (Tris) and the HPLC grade solvents acetonitrile (ACN), dimethylformamide (DMF) and tetrahydrofuran (THF). BCA (Bicinchoninic acid) protein assay kit was from Pierce Chemicals while pyroglutamyl-7-amino-4-methyl coumarin (pGlu-AMC) was sourced from Bachem. Sigma-Aldrich supplied His-Select nickel affinity gel, 7-amino-4-methylcoumarin (AMC), bovine serum albumin (BSA), Bradford reagent, dimethylsulphoxide (DMSO, HPLC grade), D,L-dithothreitol (DTT), ampicillin, IPTG, imidazole, dimethyl suberimidate (DMS), N-ethylmaleimide (NEM) and any other chemicals mentioned, which were reagent grade unless otherwise noted.

We used *E. coli* XL-10 Gold cells to express PAPI activity. Yeast extract and tryptone for the LB growth medium were from Oxoid UK. The cells contained the 5235 bp plasmid pRV5 encoding *Homo sapiens* PAPI (GenBank accession number AJ278828) as a 680 bp *rHsa-pap1*-$(His)_6$ fusion (via a Gly-Ser linker) under control of the P*tac* promoter (Vaas, 2005). This plasmid is very similar to pZK3, encoding bovine PAPI, described by Kilbane et al. (2007). The differences are located within the 680 bp segment, bounded by *Eco*R1 and *Hind*III sites, that codes for the fusion protein: an *Eco*RV restriction site at 406 bp replaces the *Eag*I site at 553 bp in pZK3 and

the corresponding human amino acid residues (in brackets) are present at positions 81 (Thr), 205 (Tyr) and 208 (Lys). Commercial DNA sequencing (MWG Biotech, Germany) verified the exact sequence of pRV5 and of the single-site PAPI mutant, Y147F.

Production and Purification of Recombinant PAPI

LB growth broth (100ml, containing ampicillin to 100μg.ml^{-1}) was aseptically inoculated with 3ml of overnight culture of *E.coli* XL-10 Gold harboring pRV5 and incubated at 37°C. Once the cells reached exponential phase (A_{600} 0.3-0.5), the inducer IPTG was added to 0.05 mM. Recombinant protein production continued for 5 h or overnight. Cells were then centrifuged (3200 × *g*), the supernatant decanted, and the cell pellet labelled and stored at 4°C until required. The pellet was re-suspended in 10ml of buffer (50mM potassium phosphate, pH 8.0). The sample was then sonicated on ice (2.5 pulses.s^{-1}, 220 W, amplitude 40, 15 min), centrifuged (3200 × *g*) to remove cell debris and the clear protein lysate supernatant was retained. Ni^{2+} resin (1ml) was added to the protein lysate, which was mixed with shaking for 1 h then poured into a column. Potassium phosphate buffer (50mM, pH 8.0, 10ml) was then run through the column ~~three times?~~. Next, wash buffer (50mM potassium phosphate, pH 8.0, containing 20mM imidazole, 20ml) was applied ~~three times?~~. Finally, elution buffer (50mM potassium phosphate buffer, pH 8.0, containing 200mM imidazole, 20ml) was run through the column ~~three times?~~ to release PAPI (wild type or mutant Y147F, as appropriate). PAPI-containing fractions were combined, mixed with glycerol (40% v/v final concentration) and stored at 4°C until required. Purified PAPI typically had a specific activity of 0.34 U.mg^{-1} (where 1 U = 1 nanomol AMC.min^{-1}.ml^{-1}) and gave a single 23-24 kDa band on denaturing gel electrophoresis (Laemmli, 1970). Specific activity of mutant Y147F was 0.83 U.mg^{-1}. Protein concentrations were measured by either the standard BCA protein assay (Smith et al., 1985) or the Bradford method (Bradford, 1976).

Quantitative Fluorimetric Measurement of PAPI Activity

The PAPI activity assay was according to Fujiwara and Tsuru (1978) as modified by Browne and Ó'Cuinn (1983). Each PAPI sample (25μL, in triplicate) was placed into separate wells of a 96-well fluorometer microplate and 100μL of the assay solution (50 mM potassium phosphate buffer, pH 8.0, containing final concentrations of 10 mM DTT, 2 mM EDTA and 0.5 mM pGlu-AMC) was added to each well. (Residual concentration of DMSO (required to prepare the 10 mM pGlu-AMC stock solution) in the assay mix did not exceed 5% v/v). The microplate was incubated at 37°C for 30 min; then, 1.5M acetic acid (100μL) was added to each well to arrest enzyme activity. Liberated AMC was measured using a Perkin Elmer LS-50 fluorescence spectrometer with excitation at 370nm and emission read at 440nm. Fluorescence readings were converted to nanomol of AMC released per minute using an AMC standard curve prepared under identical conditions. In addition to buffer, standard curves were determined in the presence of 10% (v/v) DMF, culture medium and of imidazole and also using crude and purified PAPI suspensions. Experimental values were determined using the most appropriate standard curve. The assay was shown to be linear with respect to time and amount of added PAPI (r^2 = 0.99 in both cases).

Kinetic Analysis

pGlu-AMC (10mM in DMSO) was diluted to 0.5mM with 50mM potassium phosphate buffer, pH 8.0. This solution was further diluted in buffer to give a range of pGlu-AMC concentrations and purified PAPI was assayed at each substrate concentration. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values were determined using the Enzfitter programme (Biosoft, Cambridge, UK). A similar procedure was followed for mutant Y147F.

Active site titration was based on Turk et al. (1993) but used N-Ethylmaleimide (NEM) instead of their E-64. A range of NEM concentrations (0-2.5µM, prepared by dilution of a 1mM stock solution with ultra-pure water) were used to titrate activated PAPI. At each point, 25µl of purified PAPI (diluted appropriately with 50mM potassium phosphate buffer, pH 8.0) was mixed with an equal volume of NEM solution, brought to a total volume of 100 µl with 50mM potassium phosphate buffer, pH 8.0, and incubated at 37°C for 15 min. Residual activity was then determined in triplicate. Fluorescence intensity was plotted versus NEM concentration and the operational molarity of PAPI determined. A similar procedure was followed for mutant Y147F.

Thermal Stability

Samples of purified PAPI (0.45 $mg.ml^{-1}$ stock diluted appropriately in 50mM potassium phosphate buffer pH 8.0 to yield a suitable range of fluorescence units in the activity assay) were incubated for 10 min at temperatures between 30º-80ºC, then placed on ice prior to re-warming and assay of the remaining PAPI activity in triplicate at 37°C. Buffer-only blanks were also prepared. A plot of % residual activity against temperature (°C) was constructed to give a thermal profile and the T_{50} (half-inactivation temperature) determined by inspection.

PAPI samples, prepared similarly, were placed in a waterbath held at 60ºC. Aliquots were removed at intervals, cooled rapidly on ice and the remaining PAPI activity assayed as above following re-warming. Residual activity (%, calculated from the sample's initial activity) was plotted versus time. Data were fitted to a first-order exponential decay using Enzfitter (Biosoft, Cambridge, UK) to estimate the rate constant (k) and, hence, the half-life ($t_{½}$; $0.693/k$). PAPI and mutant Y174F were tested similarly at 70ºC in buffer containing 0.12 $mg.ml^{-1}$ BSA to ensure a uniform protein concentration.

Tolerance of Organic Solvents

PAPI samples, prepared as above, were incubated for 1 h at room temperature with various solvents (ACN, DMF, DMSO, THF, methanol, ethanol) ranging in concentration from 0-90% (v/v) in 50mM potassium phosphate pH 8.0 in a final volume of 1ml. Enzyme-free blanks were also set up for each solvent at each v/v concentration. The samples were then assayed in triplicate under normal conditions and a plot of % remaining activity versus solvent concentration (% v/v) was constructed.

Chemical Modification

The protocol was based on de Renobales and Welch (1980). Purified PAPI (0.45 $mg.ml^{-1}$) was diluted with 50 mM potassium phosphate, pH 8.0 (3ml). A stock solution of dimethyl suberimidate (DMS; 2.5 $mg.ml^{-1}$) was similarly diluted and was mixed with with an equal volume of PAPI (final concentrations: DMS 25 $\mu g.ml^{-1}$; PAPI 5 $\mu g.ml^{-1}$). PAPI-DMS and a control were incubated at room temperature for 30 min. A thermal profile was then determined for each and plots of % residual activity versus temperatures were prepared.

Effects of Additives

Ammonium sulfate, trehalose and xylitol were each added to 0.5 M final concentration to separate samples of PAPI (5 $\mu g.ml^{-1}$ final concentration). Thermal profiles were determined and plots of % residual activity versus temperature prepared in each case. The effects of 10% v/v and 50% v/v glycerol were determined similarly.

Results and Discussion

Thermal Stability

The temperature profile (Figure 1) showed that PAPI activity declined above 45°C. The half-inactivation temperature ($T_{50,}$ value where observed activity was 50% of maximal) was estimated by inspection to be 60°C ± 1°C.

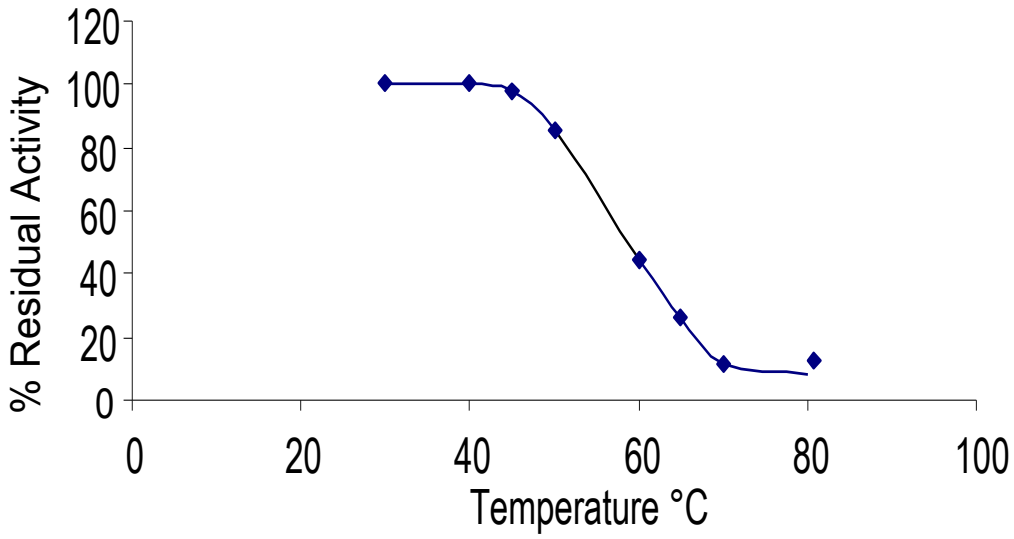

Figure 1: A temperature profile of PAPI.

Plot of % residual activity versus temperature (°C), following 10 min incubations at each temperature. Activity is represented as a percentage of the 30ºC value.

Data of % residual activity versus time at a uniform 60ºC fitted well to a first order exponential decay to yield a *k* value of 0.046 ± 0.002 min^{-1} and, hence, a half-life ($t_{½}$) of 15 min. Human PAPI expressed in Sf9 insect cells was stable at temperatures up to 40°C for 4.5 h, but activity was almost completely lost within 30 min at 60°C, in line with the present results. Maximal activity was seen at 50°C in 10 min assays (Dando *et al*., 2003), a somewhat higher temperature than our value of 45°C; the different values may arise from the use of different buffer compositions or protein concentrations. Many glycosylated polypeptides, both native and recombinant, are more heat-stable than their 'naked' counterparts expressed in *E. coli*. One potential N-glycosylation motif, Asn-Ala-Ser, occurs at positions 25-27 of human PAPI. Possibly, the insect cells used by Dando et al. (2003) perform a post-translational glycosylation of PAPI. Molecular mass values (Dando et al., 2003), however, suggest that glycosylation, if it indeed occurs, is not extensive.

Thermal stability of PAPI varies with the temperatrures preference of the source organism. The temperature-activity profile of PAPI from the hyperthermophilic archaeon *Thermococcus litoralis* has a maximum at 70°C, where it has a half-life of 1 h. Although considerably more thermostable than mesophilic enzymes, *T. litoralis* PAPI loses its activity rapidly at temperatures >80°C. This is likely to be due to destruction of the critical cysteine in the active site (Singleton *et al*., 2000). PAPI from the hyperthermophilic archaeon *T. litoralis* showed enhanced thermal stability over that of mesophilic *B. amyloliquefaciens,* probably due to the presence of inter

subunit disulphide bonds. *B. amyloliquefaciens* PAPI with an engineered inter-subunit disulphide bond showed increased thermal stability, without any decrease in enzymatic performance. However, pH stability was not altered (Kabashima *et al*., 2001). The thermophilic *Pfu*PAPI exhibits optimum activity at 90ºC (Ogasahara *et al*., 2001).

Resistance to Solvents

PAPI was unstable in most of the water-miscible, hydrophilic solvents tested. The C_{50} values (the % v/v concentration leading to half-inactivation versus aqueous buffer) for the least injurious solvents DMSO and methanol were 10% v/v and 12% v/v, respectively (errors ± 0.5 % v/v). THF, a strong denaturant, had a notably adverse effect even at 10% (v/v): activity declined sharply to about 1/10 of the aqueous level. Results with DMF were similar: at 10% (v/v) DMF, activity was 26% of that in aqueous buffer, while at 20% (v/v) DMF only 10% of the aqueous activity remained. PAPI activity declined to 21% of that in aqueous buffer at 10% (v/v) ACN. In 10% (v/v) acetone, residual activity was 33%, while above 50% (v/v) concentration, no significant PAPI activity remained. Ethanol at concentrations of 10% and 20% (v/v) reduced activity to 40% and 29%, respectively, of that in aqueous buffer. At ≥40% (v/v) ethanol, virtually no activity remained.

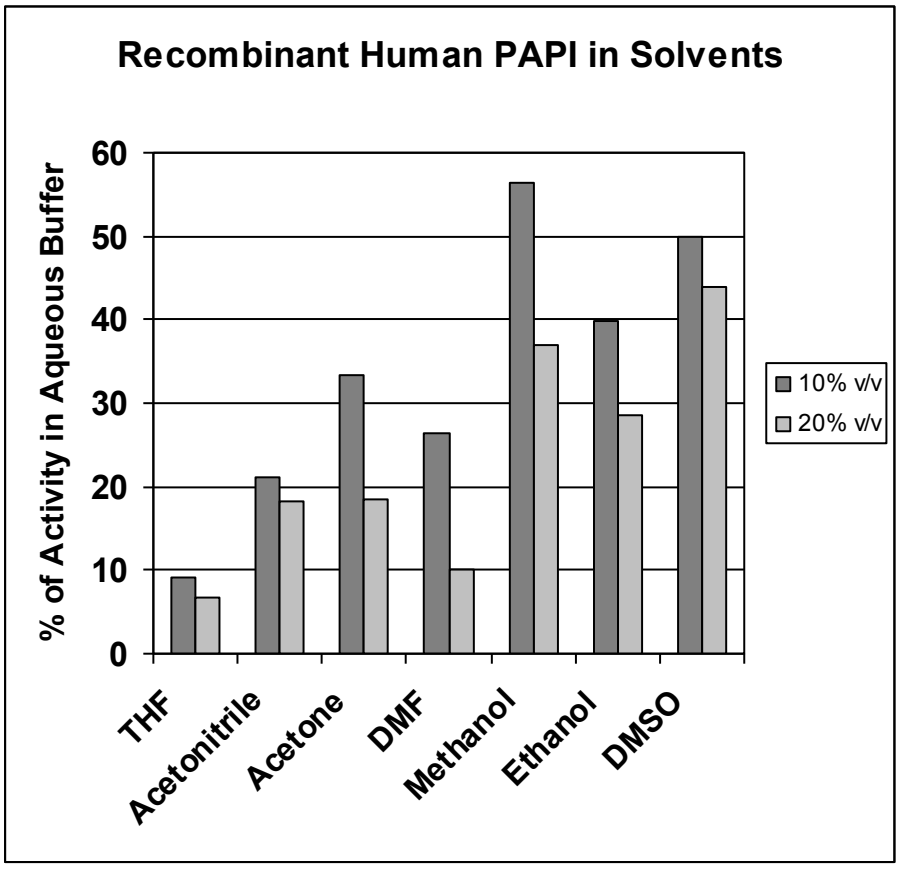

Figure 2: Effect of water-miscible solvents (10 and 20% v/v) on PAPI activity
Plot of % residual activity versus 10 or 20 (% v/v) solvents concentration.
(■) Solvents concentration 10 % (v/v), (■) solvents concentration 20 % (v/v)

Chemical Modification with Dimethyl Suberimidate (DMS)
A temperature profile indicated that chemical modification with DMS rendered PAPI *less* thermostable than native at the same concentration. Only 20% of the initial PAPI activity remained following DMS treatment, a very low recovery. This poor outcome was unexpected: DMS is a very mild protein modifying reagent which should not lead to significant inactivation. DMS treatment has effectively stabilized other enzymes such as horseradish peroxidase (HRP, 4-fold increase in $t_{½}$ at 75ºC; Ryan *et al*., 1994) and alanine aminotransferase (16-fold increase in $t_{½}$ at 35ºC; Moreno and Ó'Fágain, 1997). DMS usually reacts with Lys/ -NH_2 groups of proteins only and not with any other R-groups (Ji, 1983). There are 10 Lys residues in human PAPI, 3 of which lie within conserved sequences (R Larragy, unpublished); modification of a conserved Lys could compromise the enzyme's activity and/or stability.

Effects of Ammonium Sulfate and of Polyols on PAPI Activity and Stability
Contrary to expectation, ammonium sulfate had no stabilising or protective effect on PAPI at any of the temperatures tested. PAPI activity was significantly reduced at 0.5 M ammonium sulfate, with 35% less activity displayed than in buffer. Activity of a different brain peptidase, the proline-specific dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5) from bovine brain, was also significantly reduced (80%) in presence of 1M ammonium sulfate compared with buffer alone (Buckley, 2001).

Glycerol also inhibited PAPI: only 50% of aqueous activity was manifested in 10% (v/v) glycerol and a mere 18% activity at 50% (v/v) glycerol. Except for a marginal effect >60^{o}C at an inhibitory 50% (v/v), glycerol had no protective or stabilizing effect on PAPI at elevated temperatures.

It was hoped that PAPI would be stabilized against heat by the inclusion of xylitol or trehalose (Schein, 1990). Trehalose prevents proteins from denaturing at high temperature *in vitro*. It also suppresses the aggregation of denatured proteins, maintaining them in a partially-folded state from which they can be reactivated. The continued presence of trehalose, however, interferes with refolding (Singer and Lindquist, 1998) Trehalose (0.5M) did not stabilize PAPI.

Xylitol at 0.5M gave a significant increase in PAPI activity at 30°C and 40°C. With xylitol, PAPI retained >50% activity at 60ºC while, in its absence, the T_{50} was 45ºC. A protecting effect was also evident during thermoinactivations at 60ºC where $t_{½}$ was 60% longer with xylitol (first-order *k*-values in presence and absence of xylitol were 0.05 ± 0.002 min^{-1} ($t_{½}$ 14 min) and 0.08 ± 0.003 min^{-1} ($t_{½}$ 9 min) respectively). Note that the protein concentrations in these experiments were less than in the thermal inactivations described above, hence the different values for the PAPI controls.

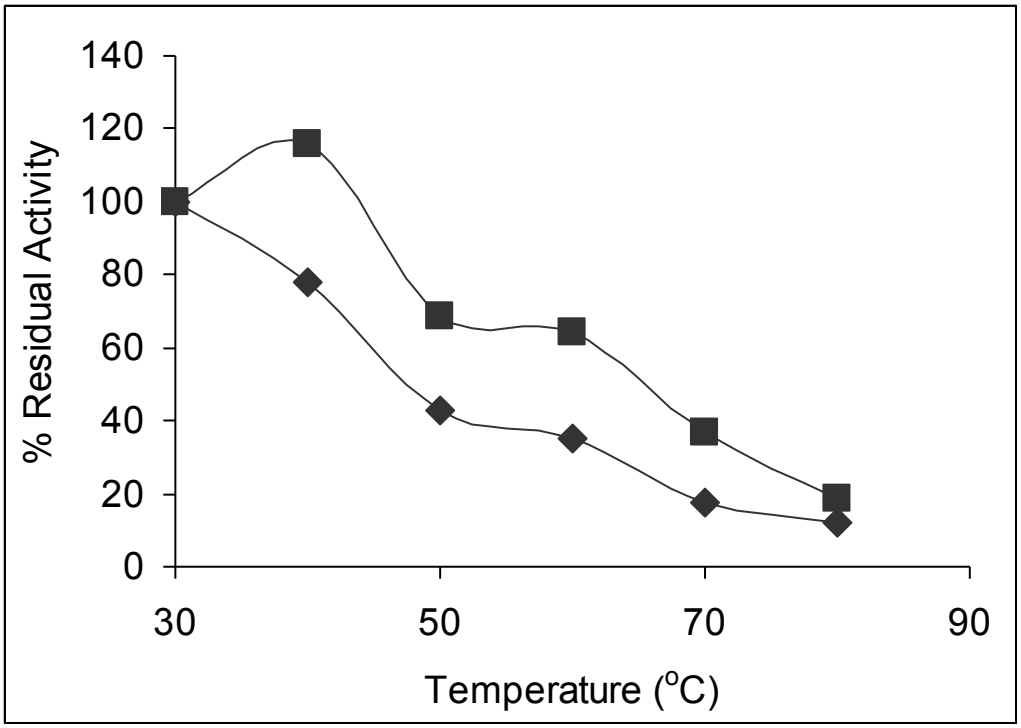

Figure 3. Effect of Xylitol on PAP1
Plot of % residual PAP1 activity versus temperature (°C). (♦) without xylitol; (■) with xylitol. Activity is represented as a percentage of the 30ºC value in each case.

Thermal Stability and pGlu-AMC Kinetics of Mutant Y147F
Tyr 147 of PAPI is located in the substrate binding site, very close to the catalytic Cys 149 (Vaas, 2005). Comparison of 13 known eukaryotic PAPI amino acid sequences shows that both of these residues lie within a conserved sequence DAGR**Y**$_{147}$L**C**$_{149}$DFTYYTSLY. At the equivalent position in prokaryotes, Phe occurs instead of Tyr in 25 of 37 sequences (R Larragy, unpublished). Both Phe and Tyr have phenyl R-groups but Tyr possesses a hydroxyl group on its benzene ring. Hydroxyls can participate in hydrogen bonding at moderate pH values or undergo ionization at strongly alkaline pH. We were curious, therefore, to investigate the effects of a Tyr147→Phe mutation.

Thermoinactivation of both wild type PAPI and Y147F (diluted appropriately in 50mM potassium phosphate buffer, pH 8.0, containing 0.12mg.ml^{-1} BSA to ensure a constant protein concentration) was studied at 70ºC over 80 min. (The higher temperature was required to achieve timely inactivation of Y147F.) Under these conditions, $t_{½}$ for Y147F was 25 min, almost 3 times longer than that of wild type PAPI (9 min; see Table 1). Remarkably, removal of the –OH group from position 147 has a very stabilizing effect on PAPI, perhaps due to improved hydrophobic packing.

Michaelis-Menten Kinetics of PAPI and Y147F on pGlu-AMC
Both wild type PAPI and mutant Y147F utilized the convenient fluorimetric substrate pGlu-AMC and displayed Michaelis-Menten kinetics. Use of NEM, a thiol-directed reagent, allowed calculation of the active sites contents [E] and, hence, calculation of the respective k_{cat} values from the experimentally-determined V_{max}, since $k_{cat} = V_{max}/[E]$. Table 1 presents the calculated values. The amidase activity showed a k_{cat}/K_m value for PAPI of 0.202 (s^{-1} M^{-1}) while the

corresponding value for Y147F was 0.212 ($s^{-1} M^{-1}$). These values are virtually identical, showing that loss of the –OH from position 147 does not affect cleavage of pGlu-AMC.

Table 1. Comparison of Native PAPI with Variant Y147F.

	Native PAPI	**Mutant Y147F**
First-order *k*-value (min^{-1}), 70°C	0.079 ± 0.003	0.028 ± 0.001
Half-life ($t_{½}$), 70°C	9 min	25 min
Protein content ($mg.ml^{-1}$)	0.12	0.12
K_m (mM)	0.13 ± 0.02	0.12 ± 0.02
V_{max} (µmol.min^{-1}.ml^{-1})	0.0013 ± 0.0001	0.0015 ± 0.0002
Active sites (µM; NEM method)	0.8	1.04
k_{cat} (s^{-1})	2.68×10^{-5}	2.45×10^{-5}
k_{cat}/K_m ($s^{-1}.M^{-1}$)	0.202	0.212

Conclusion

Themal stability of recombinant human brain PAPI proved considerably higher than would be expected for this enzyme, since mammalian pyroglutamyl peptidases normally exist at the physiological body temperature of 37°C. PAPI was catalytically active up to quite elevated temperatures, with a $t_{½}$ of 15 min at 60°C. Nevertheless, the *in vitro* stability of wild type PAPI might limit its potential applicability in peptide processing or other fields, since PAPI was unstable to most of the water-miscible organic solvents tested. Methanol and DMSO were the least injurious to PAPI activity while THF was the most deleterious. Modification of PAPI with DMS, a very mild reagent, gave only 20% recovery of initial activity and did not stabilize the enzyme. PAPI activity and stability increased with xylitol but trehalose, glycerol and ammonium sulphate conferred no benefits. Xylitol, therefore, may be a better preservative for PAPI than glycerol, the additive currently used. It would seem that chemical modification and/or additives have limited scope to enhance PAPI stability but the use of a thermophilic counterpart, or the generation of stabilized mutant variants of recombinant PAPI, should be explored further. The latter may indeed be possible, since the single-site mutant Y147F, with a $t_{½}$ of 25 min at 70°C, is notably more thermostable than wild type PAPI. With pGlu-AMC as substrate, the kinetic parameters of the two enzymes were virtually identical.

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