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REVIEW ARTICLE

Human prolidase and prolidase deficiency: an overview on the characterization of the enzyme involved in proline recycling and on the effects of its mutations

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Abstract Here we summarized what is known at the present about function, structure and effect of mutations in the human prolidase. Among the peptidases, prolidase is the only metalloenzyme that cleaves the iminodipeptides containing a proline or hydroxyproline residue at the C-terminal end. It is relevant in the latest stage of protein catabolism, particularly of those molecules rich in imino acids such as collagens, thus being involved in matrix remodelling. Beside its intracellular functions, prolidase has an antitoxic effect against some organophosphorus molecules, can be used in dietary industry as bitterness reducing agent and recently has been used as target enzyme for specific melanoma prodrug activation. Recombinant human prolidase was produced in prokaryotic and eukaryotic hosts with biochemical properties similar to the endogenous enzyme and represents a valid tool both to better understand the structure and biological function of the enzyme and to develop an enzyme replacement therapy for the prolidase deficiency (PD). Prolidase deficiency is a rare recessive disorder caused by mutations in the prolidase gene and characterized by severe skin lesions. Single amino acid substitutions, exon splicing, deletions and a duplication were described as causative for the disease and are mainly located at highly conserved amino acids in the sequence of prolidase from different species. The pathophysiology of PD is still poorly understood; we offer here a review of the molecular mechanisms so far hypothesized.

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Introduction

Extracellular and intracellular proteases perform essential functions in all living organisms by mediating non specific protein hydrolysis or acting as processing enzymes that perform highly selective, limited and efficient cleavage of specific substrates that influence many biological processes and cellular homeostasis. The study of the degradome, the complete set of proteases expressed at a specific moment or under certain circumstances by a cell, tissue or organism, has recently received a strong input for the availability of the full human genome sequence and the development of powerful tools, such as genomics, proteomics and bioinformatics. Among the 20 amino acids known to constitute proteins, proline is the most peculiar one, having its side chain cycling back to the backbone amino group and generating a pyrrolidine ring not susceptible to generic peptidase cleavage. Due to this structure, its presence effects the conformation, properties and biological functions of various molecules such as structural proteins like collagen, peptides involved in immunomodulation and coaugulation, cytokines, growth factors, neuro- and vasoactive peptides (Vanhoof et al. 1995; Yaron and Naider 1993). Only a limited number of mammalian peptidases are known to be able to hydrolyse proline adjacent bonds and their activity is influenced by the isomeric state (*cis–trans*) as well as by the position of proline in the peptide chain. All the known proline-specific peptidases cleave only when the peptide bond preceding proline has *trans* conformation. Although not directly involved in the hydrolysis of proline-containing peptides bonds, the enzyme Prolyl *cis–trans*

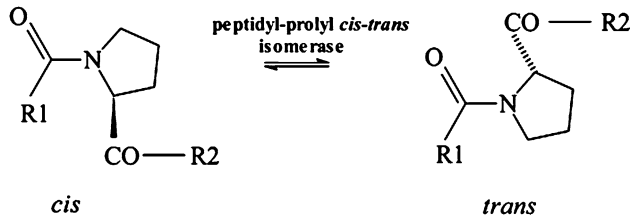

Fig. 1 X-Pro peptide in *cis* and *trans*-conformation. The reaction is catalyzed by the enzyme Peptidyl-prolyl *cis–trans* isomerase

isomerase (EC 5.2.1.8) is essential to guarantee the conversion from the *cis* to the cleavable *trans* conformation (Fig. 1) (Yaron and Naider 1993). Based on the proline position inside the proteins nine exopeptidases, one endopeptidase and two dipeptidases had been so far identified (Fig. 2). Aminopeptidase P (EC 3.4.11.9) is a prolyl specific metalloexopeptidase that cleaves any unsubstituted N-terminal amino acid adjacent to a penultimate proline residue (Cottrell et al. 2000). It was suggested to be involved in the maturation and degradation of peptide hormones, neuropeptides and in the digestion of resistant dietary proteins fragments, thus complementing the pancreatic peptidases (Blau et al. 1988). Dipeptidyl peptidase II (DPP2) (EC 3.4.14.2) is an intracellular exodipeptidase that releases an N-terminal dipeptide X–Y preferentially when Y is Ala or Pro and preferentially acts on tripeptides. DPP2 was suggested to be involved in cell differentiation, protection of cell from death and to play a role in collagen, myofibrillar proteins and small neuropeptides degradation (Maes et al. 2005, 2006, 2007; McDonald and Ohkubo 2004). Dipeptidyl peptidase IV (DPP4) (EC 3.4.14.5) is a serine exopeptidase that hydrolyzes X-Pro dipeptides from the N-terminal of proteins. It has a critical role in physiological glucose homeostasis by controlling the activity of glucagon-like peptide 1 (Marguet et al. 2000) and in the regulation of the biologic activity of multiple hormones and chemokines (Marguet et al. 2000). Recently other two Dipeptidyl peptidases DPP8 (EC 3.4.14.5) and DPP9 (EC 3.4.14.5) have been identified on the basis of their homology with DPP4 and showed similar activity (Abbott et al. 2000; Olsen and Wagtmann 2002). Leucyl aminopeptidase (EC 3.4.11.1; EC 3.4.11.5) is an exopeptidase cleaving N-terminal proline or leucine residues (Matsushima et al. 1991). Prolylcarboxypeptidase (EC 3.4.16.2) is an exopeptidase that hydrolyzes a C-terminal amino acid linked to proline in oligopeptides such as Angiotensin II and III and bradikynin (Tan et al. 1993). Carboxypeptidase P (EC 3.4.17.16) releases a C-terminal residue other than proline by preferentially cleaving a prolyl bond in molecules such as Angiotensin II and III (Hedeager-Sørensen

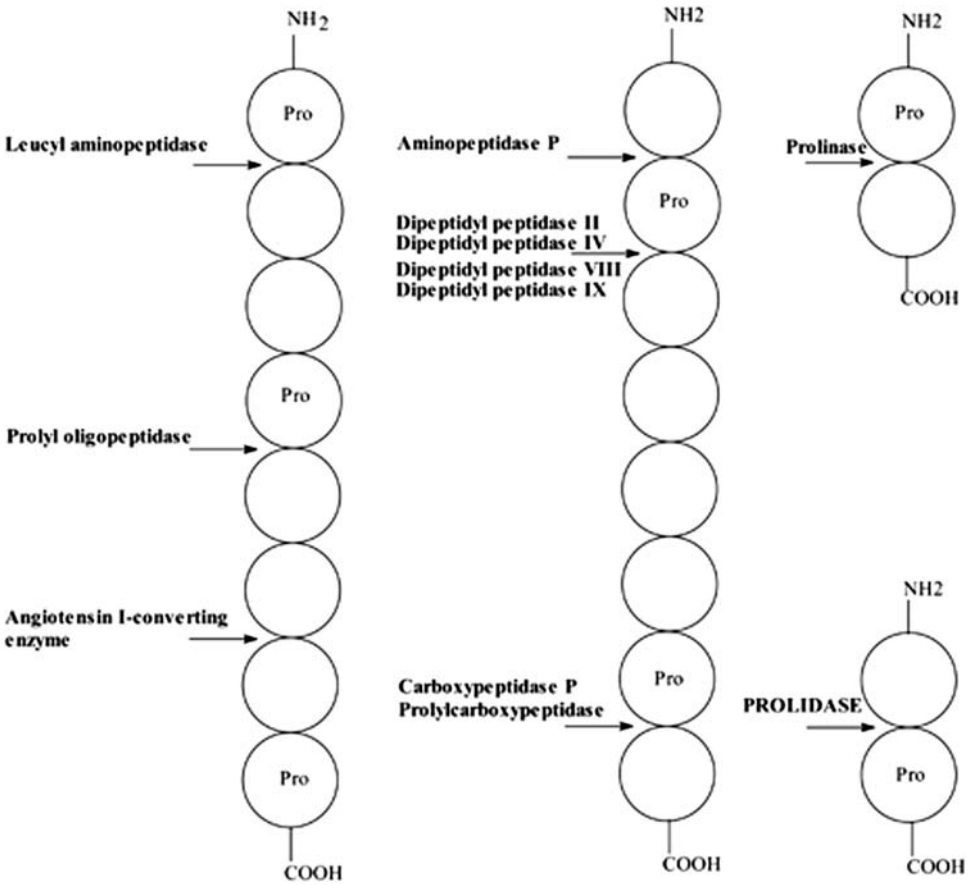

Fig. 2 Peptidases known in humans to process proline adjacent bonds

and Kenny 1985). Prolyl oligopeptidase (EC 3.4.21.26) is a cytosolic serine endopeptidase that cleaves peptide bonds at the C-terminal side of a proline residue in *trans* conformation. It is involved in the maturation and degradation of peptide hormones and neuropeptides (Fülöp et al. 1998). Another enzyme not directly involved in proline-containing peptide bonds is Angiotensin converting enzyme (EC 3.4.15.1). This exopeptidase plays an important role in blood pressure regulation and electrolyte balance by hydrolyzing Angiotensin I into Angiotensin II. The presence of proline in the peptide chain is crucial in the Renin-Angiotensin system. Renin cleaves exclusively a Leu–Leu bond in Angiotensinogen, with the requirement for a proline four residue prior to the cleavable site. The same proline prevents the degradation of Angiotensin II after its formation from Angiotensin I by Angiotensin converting enzyme, preserving its vasorestrictive capacity (Vanhoof et al. 1995). Prolinase (EC 3.4.13.8) and prolidase (EC 3.4.13.9) are the only dipeptidases able to hydrolyse the peptide bond in dipeptides containing respectively a N- or C-terminal proline or hydroxyproline residue. These enzymes play a role in collagen metabolism because of the high content of imino acids in collagen (>25%) (Royce and Steinmann 2002) and seem to be important for protein catabolism in general, but their biological functions as well as their potential use in some industry is still under investigation. We will give here an overview on what is known on prolidase function and structure as well as on the effects of prolidase mutations in humans based on our long standing interest and study of this enzyme both in vitro and in vivo systems.

Prolidase

Function

Human prolidase is a widely distributed metalloenzyme constituted by two identical subunits of Mr 54,305 with an optimum activity at pH 7.8 and 37–50°C, requiring Mn^{2+} ions for full activity (Endo et al. 1982; Richter et al. 1989; Royce and Steinmann 2002). The preferential substrate for prolidase is the Gly-Pro dipeptide (Butterworth and Priestman 1985; Endo et al. 1982, 1987; Myara et al. 1994; Nakayama et al. 2003; Oono et al. 1990; Richter et al. 1989), although the enzyme is also active against Ala-Pro, Met-Pro, Phe-Pro, Leu-Pro and Val-Pro. Its unique role is the hydrolysis of dipeptides containing a C-terminal proline or hydroxyproline residue in *trans* conformation (Vanhoof et al. 1995). Thus prolidase is involved in the latter stage of the catabolism of both dietary and endogenous proteins particularly rich in those imino acids, such as collagen, it supplies and recycles proline for proteins synthesis and cellular growth (Emmerson and Phang 1993; Yaron and Naider 1993). Beside the intracellular dipeptidase function, prolidase is also important as detoxificant against chemical agents and pesticides. Cheng et al. identified an organophosphorus acid anhydrolase that had 22% sequence identity with human prolidase and like the human enzyme catalyzes the hydrolysis of X-Pro dipeptides in presence of Mn^{2+} ions, thus being classified as prolidase (Cheng et al. 1996). Wang et al. showed that recombinant human prolidase expressed in *Pichia Pastoris* catalyzes the hydrolysis of organophosphorus compounds as well as the digestion of Gly-Pro dipeptides (Wang et al. 2006b).

Recently, the higher expression of prolidase in tumor cells and particularly in melanoma cells suggested its potential use as a viable endogenous enzyme target for selective activation of proline prodrugs (Mittal et al. 2005, 2007a, 2007b). The use of prodrugs in the treatment of various tumors has been recently successfully employed to achieve specific drug delivery, reducing the side effects of traditional chemotherapy strategies, such as unacceptable damage to normal tissues, a narrow therapeutic index and a relatively poor selectivity for neoplastic cells (Cohen et al. 2002; Druker 2002; Hvizdos and Markham 1999; Miwa et al. 1998; Venturini 2002). In particular, in vitro and in vivo studies demonstrated the effectiveness of such approach for delivering to melanoma cells the prophalan-L drug. Prophalan is a melphalan prodrug obtained by linking the carboxyl terminal end of melphalan to the amino group of a proline residue and thus hydrolysable by endogenous prolidase (Mittal et al. 2005).

Prolidase has also an important biotechnological application in the dietary industry. Dipeptides containing proline cause bitterness in cheese, thus prolidase can be used to reduce cheese bitterness by releasing proline from the dipeptides (Bockelmann 1995).

Structure

Prolidase in eukaryotic cells is post-translationally modified by glycosylation and phosphorylation. Based on its primary structure human prolidase contains two putative site for N-glycosylation (N13 and N172; NetNGlyc1.0 Server) and one putative site for O-glycosylation (T458, NetOGlyc3.1 Server). In fact, about 0.5% of carbohydrate content was reported by Sjostrom et al. based on electrophoretic separation and staining of the gels respectively by Schiff's reagent and Coomassie Blue (Sjostrom and Noren 1974). The nature of the glycosidic chains are unknown as well as their role since recombinant human prolidase obtained in prokaryotic host (*E. Coli*) showed activity similar to the endogenous eukaryotic one (Lupi et al. 2006a). Based on Tyr, Thr and Ser phosphorylation consensus sequence, the human prolidase has four Ser (109, 134, 198, 236, Score

> 0.9), one Thr (86, Score > 0.9) and two Tyr (117, 124, Score > 0.9) putative sites for phosphorylation (NetPhos2.0 Server) (House et al. 1984; Songyang et al. 1996; Yaffe and Smerdon 2004). Surazynski et al. (2001, 2005) demonstrated that prolidase is both a phosphotyrosine and a phosphothreonine/serine enzyme. Both phosphorylations, mediated respectively by Mapk pathway and NO/cGMP signaling, upregulate prolidase activity. The structure of human prolidase, as well as the composition of its active site and the catalytic mechanism, are still poorly understood. Until recently, the only information available was based on the sequence homology between human and *Pyrococcus furiosus* prolidase (Pfprol) (25% identity and 43% similarity, Fig. 3) for which the complete structure and active site organisation were described in details by crystallographic and site directed mutagenesis studies both in native and *E. Coli* recombinant enzyme (Du et al. 2005; Ghosh et al. 1998; Maher et al. 2004; Royce and Steinmann 2002). The prolidase subunit defined in Pfprol has an N-terminal domain (domain I, residues 1–112), an helical linker (residues 113–123) and a C-terminal domain (domain II, residues 124–348). Although the prolidase in *Pyrococcus* is active in presence of cobalt ions, in the crystal structure they were substituted by zinc ions. Pfprol is a homodimer having one Co-bound dinuclear metal cluster per monomer with one tightly bound (Co1) and one loosely bound (Co2) cobalt site (Du et al. 2005). The active site in Pfprol has been identified in an oval depression on the inner (concave)

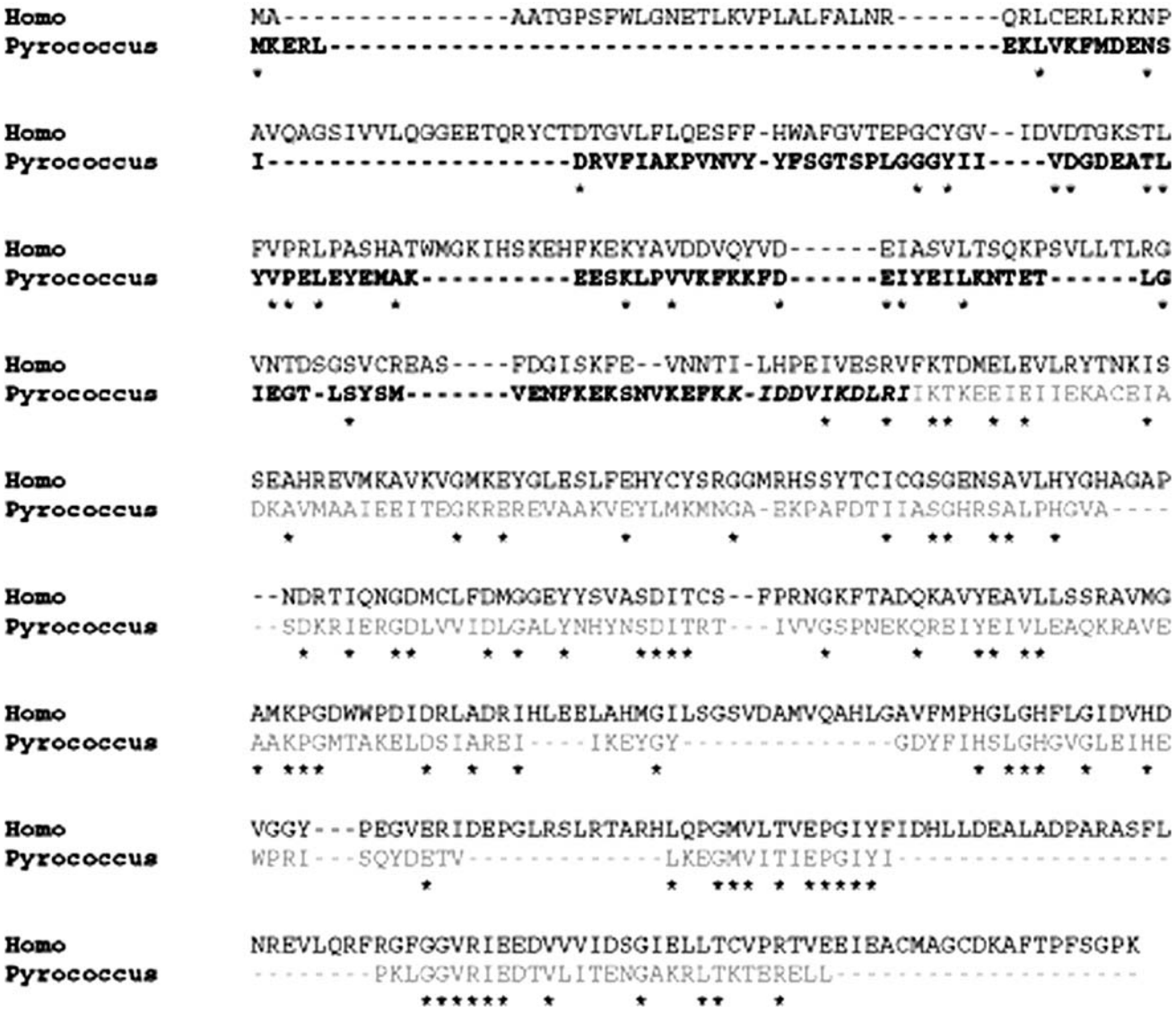

Fig. 3 Amino acid sequences alignment for prolidase from *Homo sapiens* and *Pyrococcus furiosus*. In the *Pyrococcus furiosus* sequence in *bold* are indicated amino acids defining domain I, in *italic bold* those defining the helical linker, in *grey* domain II. The *asterisks* indicate the amino acids conserved among the two species

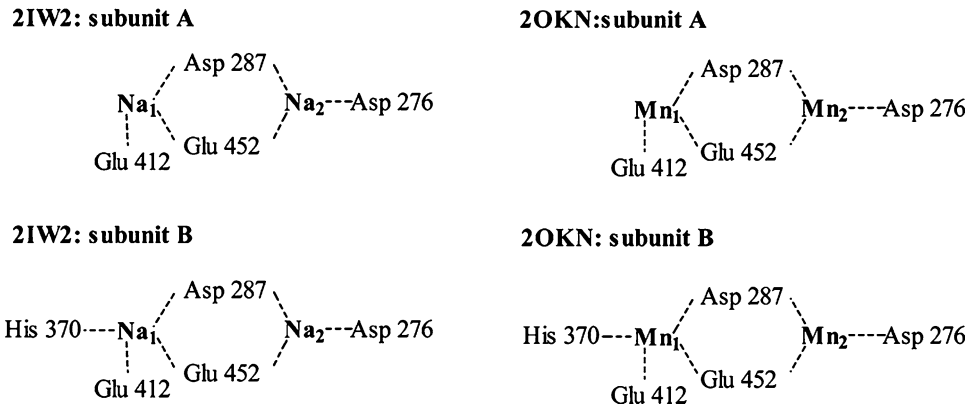

Fig. 4 Dinuclear metal centre of human prolidase. Subunits A and B of 2IW2 prolidase containing Na^+ ions (*left panels*); subunits A and B of 2OKN prolidase containing Mn^{2+} ions (*right panels*)

surface of the curved β-sheet of domain II (the so called "pita bread") (Maher et al. 2004). X-ray crystal structure analysis of *P. furiosus* and mutagenesis studies identified 5 amino acids that function as metal-binding residues: His284 and Glu313 solely bind to the first cobalt centre (Co1), Asp209 to the second cobalt centre (Co2), and Asp220 and Glu327 to both Co^{2+} ions (Du et al. 2005; Maher et al. 2004; Willingham et al. 2001). Only the crystal of one monomer had been described by the authors since the second was seriously disordered. Although no papers have been published yet, two crystal structures for human prolidase are available on Protein Data Bank, the first containing five Na^+ ions, four organized in two dinuclear centres and one located in an external position of the homodimer (2IW2) (Mueller et al. Protein Structure factory, unpublished, http://www.pdb.org), the second with four Mn^{2+} ions organized in two dimetal clusters (2OKN) (Mueller et al. Protein Structure factory, unpublished, http://www.pdb.org). In general, both structures confirm what observed in *P. furiosus* crystal regarding the active metal centre (Fig. 4). Both 2IW2 and 2OKN structures are homodimers with each subunit containing two ions, Na^+ and Mn^{2+}, respectively. In subunit A 4 amino acids are in a region closer to the metal ions and potentially able to interact with them: binding to the first ion (Mn1/Na1): Glu412 (Glu313 in Pfprol); binding to the second ion (Mn2/Na2): Asp276 (Asp209 in Pfprol); binding to both ions: Asp287 and Glu452 (Asp220 and Glu327 in Pfprol). Subunit B, where 5 amino acids were identified in the metal centre surrounding region, is identical to subunit A with the relevant exception of His370 (His284 in Pfprol) binding to the first ion (Mn1/Na1). In the metal cluster of the B subunit of both structures Thr289 is closed to the second ion (Mn2/Na2); in 2OKN, Thr410 is closed to Mn1. Both Thr289 and Thr410 are highly conserved in the prolidase from different organisms. The fifth Na^+ ion in 2IW2 is close to Gly318, Ala319, Met320 and Lys321; Lys321 is highly conserved (Mueller et al. Protein Structure factory, unpublished, http://www.pdb.org). The mechanism of prolidase catalysis is still unknown although similar mechanisms had been reported for the "pita bread" of different metalloenzymes (Maher et al. 2004; Roderick and Matthews 1993). Since they all share the same set of conserved residues at the active site and act upon similar substrates, it had been proposed they share a conserved catalytic mechanism. Briefly, the required proline residue is bound in a hydrophobic pocket which correctly orients the substrate for catalysis. A solvent molecule, polarized by interacting to one or both metal ions, is positioned for nucleophilic attack on the carbonyl carbon of the scissile peptide bond. A conserved Glu will abstract a proton from this nucleophile. An His residue and one of the ion will stabilize the intermediate following the nucleophilic attack and in the final step the intermediate will be converted to product thanks to the donation of the abstracted proton from the Glu to the amine leaving group (Copik et al. 2003; Graham et al. 2006; Lowther and Matthews 2002; Wilce et al. 1998).

Recombinant prolidase

Recombinant proteins are useful tools to investigate proteins structure and function and to develop new therapeutic strategies (Buckel 1996). Eukaryotic proteins are often post-translationally modified, thus requiring eukaryotic host when expressed as recombinant molecules. Unfortunately, eukaryotic expression is expensive and gives low protein yield forcing the researchers to use prokaryotic systems, which require extensive biochemical evaluation to validate the biological properties of recombinant proteins. Recombinant prolidase was expressed and purified from both eukaryotic, yeast and Chinese Hamster Ovary (CHO) cells, and prokaryotic hosts, *E. Coli* (Lupi et al. 2006a; Wang et al. 2005, 2006b) (Table 1). The recombinant enzyme obtained from *S. Cerevisiae*, *Pichia Pastoris* and CHO was isolated to purity by different chromatographic techniques depending on the expression vectors used for the transfection. In yeasts a combination of ion-exchange and gel

Table 1 Biochemical properties of recombinant prolidase from eukaryotic and prokaryotic hosts and of endogenous enzyme obtained from different tissues

Recombinant protein	Host	Preferential substrate	Optimal pH	Optimal temperature (°C)	Ion required for activity	Molecular mass		Ref.
						Monomer (kDa)	Dimer (kDa)	
	Eukaryotic							
	S. Cerevisiae	Gly-Pro	8	50	Mn^{2+}	56		(Wang et al. 2005)
	P. Pastoris					73		(Wang et al. 2006b)
	CHO	Gly-Pro	7.8	50	Mn^{2+}	58		(Lupi et al. 2006a)
	Prokaryotic							
	E. Coli	Gly-Pro	7.8	50	Mn^{2+}	57	123	(Lupi et al. 2006a)

Endogenous protein	Tissue	Preferential substrate	Optimal pH	Optimal temperature (°C)	Ion required for activity	Molecular mass		Ref.
						Monomer (kDa)	Dimer (kDa)	
	Red blood cells	Gly-Pro	7.8	37–50	Mn^{2+}	55–58	97	(Endo et al. 1982; Endo et al. 1987; Liu et al. 2005; Richter et al. 1989)
	Kidney	Gly-Pro	7.8	37	Mn^{2+}	55	115	(Myara et al. 1994)
	Liver					56	97	(Endo et al. 1987)
	Fibroblast	Gly-Pro	7.8	37–48–50	Mn^{2+}	58	105	(Boright et al. 1989; Butterworth and Priestman 1985; Nakayama et al. 2003; Oono et al. 1990; Priestman and Butterworth 1984)

filtration chromatography was used, in CHO an affinity chromatography using a Ni^{2+} loaded resin was applied, taking advantage of the presence of an histidine tag, removed after protein purification by specific enterokinase digestion. The recombinant prolidase obtained from the different eukaryotic hosts showed Gly-Pro as preferential substrate, an optimum activity at pH 8.0, an increase of activity from 20 to 50°C, with a loss of activity at higher temperatures and required the presence of Mn^{2+} ions for full activity, as for the endogenous enzyme (Boright et al. 1989; Butterworth and Priestman 1985; Endo et al. 1982, 1987; Liu et al. 2005; Myara et al. 1994; Nakayama et al. 2003; Oono et al. 1990; Priestman and Butterworth 1984; Richter et al. 1989). Interestingly, the recombinant enzyme obtained from *S. Cerevisiae* and from CHO showed a molecular mass of 56 and 58 kDa respectively, in agreement with the size reported in literature for the human prolidase and compatible with a reported 0.5% glycosylation of the enzyme (Boright et al. 1989; Endo et al. 1988; Richter et al. 1989), whereas the enzyme purified from *P. Pastoris* showed a molecular mass of 73 kDa that the authors attributed to an higher extent of glycosylation without further investigations (Wang et al. 2006b). An high amount of recombinant human prolidase was obtained in *E. Coli* (8 mg/L of bacterial culture able to hydrolyze 18 g Gly-Pro/h) by means of affinity chromatography using a Ni^{2+} resin followed by digestion with Factor Xa to remove the histidine Tag. The biochemical properties of such enzyme in terms of substrate specificity, optimum pH and temperature of activity and ion dependence were identical to the ones described both for endogenous enzyme and recombinant prolidase from eukaryotic hosts (Lupi et al. 2006a). Due to the higher amount of purified enzyme it was possible to perform N-terminal sequence analysis as well as mass spectrometry analysis to confirm its identity and purity (Fig. 5). Also its molecular mass in native homodimeric (123 kDa) and monomeric (57 kDa) forms was evaluated by gel filtration chromatography (Fig. 6). Further investigation showed that the recombinant enzyme from prokaryotic host can be stabilized and kept active at 37°C by addition of 0.75 mM reduced glutathione (GSH) and 1 mM $MnCl_2$ with or without 25% PEG up to 3 days and in presence of GSH and Mn^{2+} and 25% glycerol up to 6 days (Fig. 7).

Prolidase deficiency

The human prolidase gene (Peptidase D, PEPD, AC_008744) is located on chromosome 19 (19p12–p13.2) and contains 15 short exons, ranging from 45 bp (ex 7) to 528 bp (ex 15) and 14 very large introns ranging from 1.1 Kb (int 8) to >50 Kb (int 9), overall spanning more than 200 kb

N - terminal sequence

HMAAATGPSFWLGNETLKVPLALFALNRQRLCERLRKNPAV

Mass Spectrometry analysis

Protein Name	Acc number	Mw	pI	Identified Peptides	Sequence Coverage
PEPD_HUMAN	P12955	54417	5.64	20	33%

-aaatgpsfw lgnetlkvpl alfalnr**QRL CER**lr**KNPAV QAGSIVVLQG GEETQRYCTD TGVLFR**qesf fhwafgvtep
gcygvidvdt gk**STLFVPR**l pashatwmgk **IHSKEHFKEK** yavddvqyvd eiasvltsqk psvlltlr**GV NTDSGSVCRE**
ASFDGISKFE VNNTILHPEI VECRVFKTDM ELEVLRYTNK ISSEAHRevm kavkvgmkey eleslfehyc ysrggmrhss
ytcicgsgen savlhyghag apndrtiqng dmclfdmgge yycfasditc sfpangkfta dqk**AVYEAVL RSSR**avmgam
kpgvwwpdmh rladrihlee lahmgilsgs vdamvqahlg avfmphglgh flgidvhdvg gypegverid epglrslrta
rhlqpgmvlt vepgiyfidh lldealadpa rasflnrevl qr**FRGFGGVR IEEDVVVTDS GIELLTCVPR** tveeieacma
gcdk**AFTPFS GP**

Fig. 5 N-terminal sequence and mass spectrometry analysis of recombinant human prolidase from *E. Coli*. *Panel a*: the first 25 amino acids (in *bold*) were identified by N-terminal Edman degradation performed on a Hewelett Packard sequencer G1000A. *Panel b*: 20 tryptic peptides of recombinant enzyme were identified as human prolidase with a 33% of the sequence coverage (http://www.expasy.org/tools/aldente)

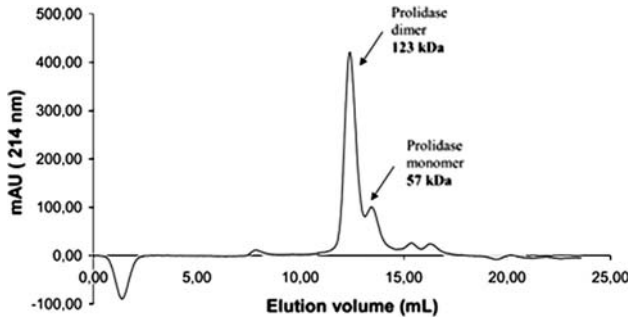

Fig. 6 Gel filtration chromatography of recombinant human prolidase from *E. Coli*. Purification on Superose 12 (Amersham Biosciences) with 10 mM Tris–HCl, 0.57 mM DTT, 0.3 M NaCl, pH 7.8 as elution buffer was performed using HPLC. Molecular weight measured for monomeric and dimeric prolidase were evaluated by means of the following standards: thyroglobulin (molecular mass: 689 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (63.5 kDa), ovalbumin (48.1 kDa), chymotrypsinogen (20.4 kDa) at 214 nm

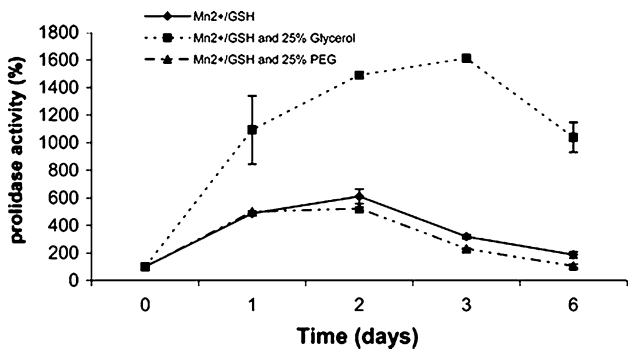

Fig. 7 Activity of recombinant human prolidase from *E. Coli* after long-term incubation at 37°C in the presence of GSH and Mn^{2+} (*diamonds*); GSH, Mn^{2+} and 25% glycerol (*squares*) and GSH, Mn^{2+} and 25% PEG (*triangles*). The activity on day 0 was assumed to be 100%. A triplicate was performed for each experiment

of genome sequence (Tanoue et al. 1990b). The mRNA (1.8 kb, NM_000285) encodes a polypeptide of 493 amino acids (Endo et al. 1989). Mutations in prolidase gene causing the reduction or the loss of prolidase activity are responsible for PD (OMIM 170100), an extremely rare autosomal recessive disorder of the connective tissue with an estimated incidence of 1–2 cases per million births (Royce and Steinmann 2002). The frequency of PD is probably underestimated due to physicians unfamiliarity with this condition; moreover, the frequency might be dependent on the population considered, as it is often the case for recessive disorders. PD patients of various ethnic origin had been reported, but due to the small number of patients is not yet possible to identify founders regions and hot spot causative allele for particular geographic places (Fig. 8, Table 2).

Clinical phenotype

The phenotypic spectrum of patients with PD is very wide although it is possible to delineate few recurrent clinical outcomes such as mild to severe skin ulcerations, recurrent infections of the respiratory tract and different degree of mental retardation. The untractable lesions, often located at the lower limbs and responsible for extremities amputation in the more severe cases, are frequently preceded by other dermatological manifestations that may occur anywhere and include erythematous papular eruptions, telangiectasias with pruritus and photosensitivity, impetigo-like eruptions, pruritic eczematous lesions and necrotic papules. Facial dysmorphism is also frequent in PD patients which present low hairline and facial hirsutism, a saddle nose, ocular hypertelorism, micrognathia, a high arched palate, mandibular protrusion and exophthalamus. Other common clinical features are splenomegaly, hypotonia, skeletal anomalies and hypergammaglobulinemia. In at least three PD cases a systemic lupus erythematosus diagnosis was made, but the link between the two diseases is not completely understood (Bissonnette et al. 1993; Di Rocco et al. 2007; Royce and Steinmann 2002; Shrinath et al. 1997). The manifestations in PD patients are usually detectable after birth or in early childhood, but late-onset cases have also been reported and the basis of this delay in clinical outcome is still an open question mark in the understanding of this genetic disorder (Dyne et al. 2001). The metabolic hallmarks of PD are iminodipeptiduria and lack of or

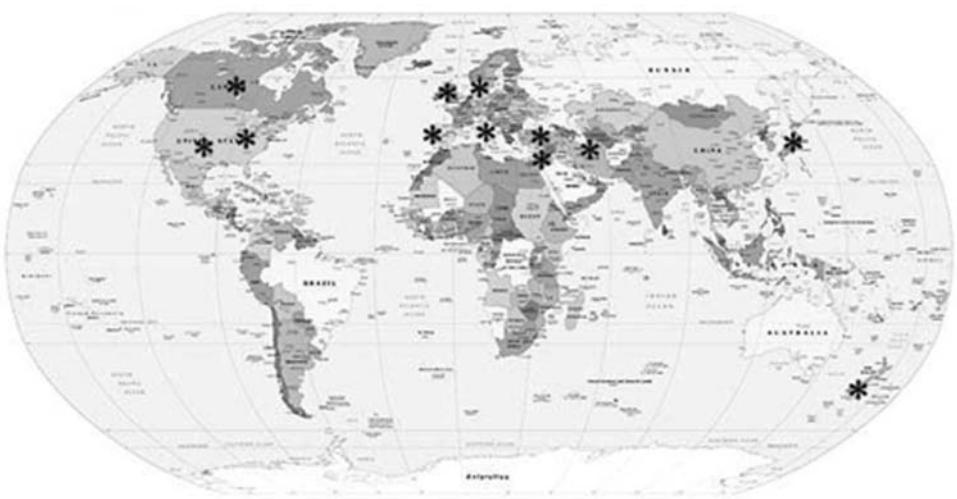

Fig. 8 World map showing the ethnic origin (indicated with *asterisks*) of the PD patients molecularly characterized

reduced prolidase activity in erythrocytes, leukocytes or cultured fibroblasts; two recent reviews exhaustively describe the biochemical diagnostic techniques used for PD patients (Kurien et al. 2006; Viglio et al. 2006). The characterization of the molecular defect by full prolidase genomic DNA sequence is ultimately necessary for prenatal diagnosis and for further confirmation of the diagnosis (Lupi et al. 2006b). No definitive cures are available so far, but oral supplementation with manganese, a cofactor of prolidase, and vitamin C, acting on collagen synthesis, have been attempted with different success (Lapiere and Nusgens 1969; Sheffield et al. 1977). Also, blood transfusions and aphaeresis (Berardesca et al. 1992; Endo et al. 1982; Hechtman et al. 1988; Lupi et al. 2002), corticosteroid treatment (Shrinath et al. 1997; Yasuda et al. 1999), topical application with growth hormone (Monafo et al. 2000), topical antibiotics for the skin lesions (Ogata et al. 1981) and ointment with Gly and Pro (Arata et al. 1986; Jemec and Moe 1996) have been tested. More recently the efficacy of prolidase-loaded liposomes in restoring normal prolidase activity in vitro cultured fibroblasts from patients with PD was described (Perugini et al. 2005).

Molecular characterization

Among over 60 patients described in literature (Royce and Steinmann 2002), only 30 were molecularly characterized (Table 2) and only 17 mutant alleles had been reported in homozygosis or compound heterozygosis as causative for the disease: eight point mutations, six causing amino acids substitution and two generating premature stop codon; three intron donor and two intron acceptor mutations responsible for exon splicing defects; two small and one very large deletions causing respectively a missing amino acid or the lack of exon 14; one small duplication responsible for a truncated enzyme due to an out of frame defect with introduction of stop codon (Fig. 9). The analysis of the mutations involving a single amino acid shows that six causative point mutations and one small deletion occur in amino acids well conserved in all other known prolidase sequences (Maher et al. 2004) and that were shown to be relevant for the structure or function of the prolidase from the archeon *Pyrococcus Furiousus* based on crystallographic and mutagenesis studies: Arg184Gln, (Arg122 in Pfprol), Gly278Asp (Gly211 in Pfprol) and Gly448Arg (Gly323 in Pfprol) with structural functions and Asp276Asn (Asp209 in Pfprol), Glu412 (Glu313 in Pfprol) and 452delGlu (Glu327 in Pfprol) all relevant for the cofactor metal binding. Ser202Phe (Ala140 in Pfprol) substitution and 231delTyr (Lys170 in Pfprol) are not highly conserved amino acids, but are located in the "pita bread" region potentially affecting the structure and function of the enzyme. Arg184X, an highly conserved amino acid, and Arg265X are both responsible for the generation of a truncated form of the enzyme lacking the C-terminal part where the catalytic site is supposed to be located (Kikuchi et al. 2000; Wang et al. 2006a). The intronic point mutation at the acceptor site of intron 4 (IVS4-1G>C) causes the deletion of 16 amino acids of exon 5 (132–147, P12955); inside this exon, Ile132 and Leu136 are highly conserved among different species: *Homo Sapiens*, *Mus Musculus*, *Suberites domuncula*, *Lactobacillus delbrueckii*, *Pyrococcus furiosus* and *Homo Sapiens*, *Mus Musculus*, *Emericella Nidulans*, *Pseudoalteromonas haloplanktis* and *Pyrococcus furiosus*, respectively. The point mutation IVS6-1A>G at the acceptor site of intron 6 causes the deletion of the 15 amino acids of exon 7 (169–183) causing the substitution of Lys168Asn in exon 6 and the lack of the highly conserved Ile180. The two point mutations at the donor site of intron 11 (IVS11 + 1G>C and IVS11 + 1G>A) are responsible for the splicing of exon 11 (248–273, 26 amino acids) that contains the highly conserved Ala252 and His255 residues. The 13 bp duplication in exon 8 generates a premature stop

Table 2 List of the molecular characterized PD patients

Patient ID	Exon	Intron	Mutation	Effect on protein	Prolidase activity[h](%)	Clinical phenotype			Ethnic origin	Ref.
						Skin ulcers	Mental retardation	Infections		
1(WG1298)		4	IVS4-1G>C/Null allele	Delex 5	≈8[i]	+	+	+	Oklahoma	(Ledoux et al. 1994)
2(WG1625)		6	IVS6-2A>G/IVS6-2A>G	Delex 7	<1[j]	+	+	−	Canada	(Ledoux et al. 1994)
3[a]		7	IVS7-1G>A/IVS7-ss1G>A	Alternative splicing	<9[i]	+	+	−	Italy	(Forlino et al. 2002)
4[a]		7	IVS7-1G>A/IVS7-1G>A	Alternative splicing	<9[i]	+	−	−	Italy	(Forlino et al. 2002)
5	8		611duplAGGCCCACCGTGA/ 611duplAGGCCCACCGTGA	Fs and premature stop	<10[i]	+	+	+	Turkey	(Lupi et al. 2006b)
6(WG1077)	8/12		551G>A/833G>A	Arg184Gln	≈8[i]	−	−	−	Canada	(Ledoux et al. 1996)
7	8		551C>T/551C>T	Arg184X	None[i,j]	+	+	+	Japan	(Kikuchi et al. 2000)
8	8		605C>A/605C>A	Ser202Phe	None[i,j]	−	+	−	Middle East	(Hershkovitz et al. 2006)
9[b]	10		691delTAC/691delTAC	231delTyr	≈5[i]	+	−	−	Portugal	(Lupi et al. 2004)
10[b]	10		691delTAC/691delTAC	231delTyr	≈5[i]	+	−	−	Portugal	(Lupi et al. 2004)
11		11	IVS11 + 1G>C/Null allele	Delex 11	<9[i]	+	+	−	Italy	(Forlino et al. 2002)
12		11	IVS11 + 1G>A/IVS11 + 1G>A	Alternative splicing	<9[j]	−	−	+	India	(Morel et al. SSIEM Germany 2007)
13[c]	11		793C>T/793C>T	Arg265X	<1[j]	+	−	+	Ohio	(Wang et al. 2006a)
14[c]	11		793C>T/793C>T	Arg265X	<1[j]	+	−	+	Ohio	(Wang et al. 2006a)
15[c]	11		793C>T/793C>T	Arg265X	<1[j]	+	−	+	Ohio	(Wang et al. 2006a)
16[c]	11		793C>T/793C>T	Arg265X	<1[j]	+	−	+	Ohio	(Wang et al. 2006a)
17[d]	12		826G>A/826G>A	Asp276Asn	<5[i]	+	−	−	Middle East	(Tanoue et al. 1990a)
18[d]	12		826G>A/826G>A	Asp276Asn	<5[i]	+	−	−	Middle East	(Tanoue et al. 1990a)
19	12/14		826G>A/1342G>A	Asp276Asn	<2[i]	+	−	−	Denmark	(Lupi et al. 2006b)
20	12/14		833G>A/1342G>A	Gly278Asp	<3[i]	+	−	+	Denmark	(Lupi et al. 2006b)
21[e]	14		1234G>A/1234G>A	Glu412Lys	None[j]	+	−	−	Turkey	(Lupi et al. 2006b)
22[e]	14		1234G>A/1234G>A	Glu412Lys	None[j]	−	−	−	Turkey	(Lupi et al. 2006b)
23(WG1343)	14		1342G>A/Null allele	Gly448Arg	<2[i]	+	+	+	Canada	(Ledoux et al. 1994)
24(WG1194)	14		1342G>A/1342G>A	Gly448Arg	<7[i]	+	+	+	UK	(Ledoux et al. 1994)
25[f]	14		1342G>A/1342G>A	Gly448Arg	<10[i]	+	+	+	Italy	(Forlino et al. 2002)
26[f]	14		1342G>A/1342G>A	Gly448Arg	<10[i]	+	−	−	Italy	(Forlino et al. 2002)
27	14		1342G>A/1234G>A	Gly448Arg	None[i]	+	+	−	Italy	(Lupi et al. 2006b)
28[g]	14		del774bp	Delex14	None[i]	+	+	−	Japan	(Tanoue et al. 1991)
29[g]	14		del774bp	Delex14	None[i]	+	−	−	Japan	(Tanoue et al. 1991)
30(WG1082)	15		1354delGAG/Null allele	452delGlu	<5[i]	+	+	−	Australia	(Ledoux et al. 1994)

In parenthesis: the original cell lines names, when available [a]Unrelated, [b]Unrelated, [c]Family related, [d]Unrelated, [e]Sisters, [f]Brothers, [g]Sisters, [h] The prolidase activity is expressed as percentage with respect the normal values, [i]Prolidase activity measured in fibroblasts, [j]Prolidase activity measured in serum

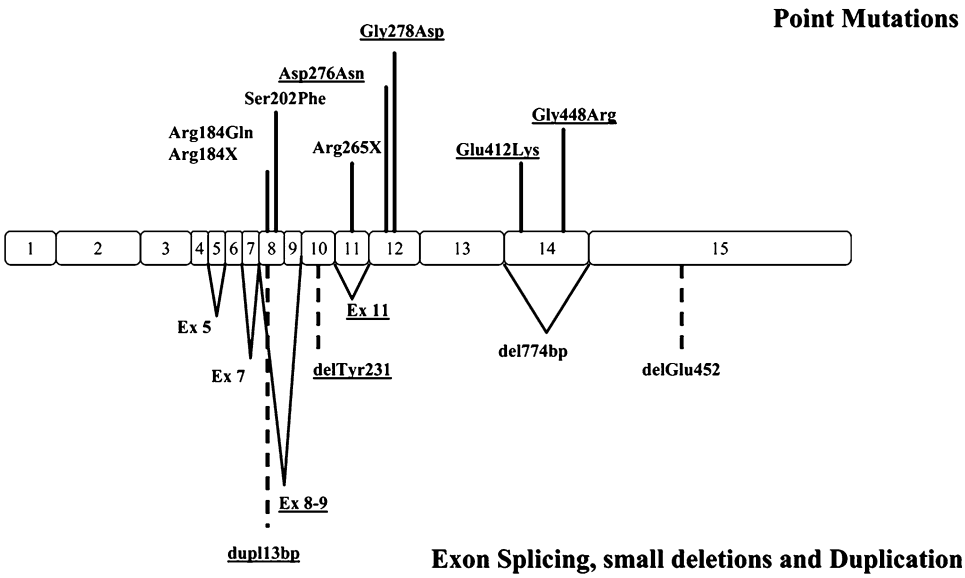

Fig. 9 Complete map of the known PEPD mutant alleles causing PD. Point mutations on *top* are indicated with the amino acid position at which they occur and the type of residue substituted. Exon splicing, small deletions and duplication are indicated on the *bottom*. Mutations identified in our laboratory are *underlined*

codon after 18 amino acids from the insertion site and results in the synthesis of a truncated and not active prolidase (Lupi et al. 2006b). The genomic deletion of 774 bp (Tanoue et al. 1991) eliminates the whole exon 14, that include the 2 highly conserved amino acids Glu412 and Gly448, whose mutations is also causative for PD. Furthermore the lack of exon 14 caused the synthesis of a shorter and inactive prolidase of Mr 49,000.

Genotype-phenotype correlation

Since phenotype, age of onset and clinical course of PD are very variable, even within the same family, and the number of molecularly characterized patients is very small, it is still difficult to define a genotype–phenotype relationship for this disease. Thus any attempts to correlate enzyme activity, evaluated in blood or cultured fibroblasts, amount of secreted urinary dipeptides or/and intracellular dipeptides accumulation in cultured fibroblasts with a specific mutation or clinical outcome were not successful (Forlino et al. 2002; Lupi et al. 2004).

In addition as for many other genetic disorders, also for PD the presence of phenotypic variability was described. In fact patients with the same mutation showed different clinical phenotype, in particular we recently described two sisters with the same molecular defect (Glu412Lys), but one with the typical PD symptoms and the other asymptomatic (Lupi et al. 2006b).

Pathophysiology of prolidase deficiency

As stated above, the main clinical outcomes in PD patients are extensive skin ulcerations, defective wound healing and frequent infections (Royce and Steinmann 2002).

The molecular basis of such phenotype, starting from the lack of prolidase activity, is still poorly understood although few hypotheses were proposed.

Forlino et al. suggested that the typical skin lesions in PD patients could be due to a necrosis-like cellular death. Such feature was identified in long term culture fibroblasts obtained from PD patients skin biopsy. The authors correlated the necrosis to a significant accumulation of undigested Gly-Pro dipeptides responsible for osmotic cellular stress and consequent ruptures of the plasma membrane (Forlino et al. 2002). Furthermore, in long-term cultured PD fibroblasts the mitochondria appeared swollen with a light matrix, peripheral scattering of cristae and loss of membrane potential confirming the activation of a necrosis-like event (Schweichel and Merker 1973). The release of the cytoplasmic contents into the surrounding tissues following cellular death might cause an inflammatory response (Desagher and Martinou 2000) probably underlying the ulcers and infections of PD patients.

It has been recently hypothesized that lack of prolidase could be responsible for delayed wound healing through an abnormal nitric oxide (NO) signalling pathway. NO is a free radical, which is involved in various cellular processes (Koshland 1993; Surazynski et al. 2005) including collagen metabolism and matrix degradation and in particular is highly expressed in tissues undergoing repair (Surazynski et al. 2005). It is produced by immuno cells at the site of the inflammation and it stimulates the local fibroblasts to produce NO by themselves (Giustizieri et al. 2002; Marcinkiewicz 1997; Marcinkiewicz and Chain 1993; Marcinkiewicz et al. 1996; Schäffer et al. 1997). NO also regulates the expression of multiple cytokines which are activated or inactivated by DPP4 and its homologues whose activity is inhibited by X-Pro dipeptides (Hechtman

2001). In addition NO stimulates collagen synthesis (Busek et al. 2003; Flentke et al. 1991).

Recently, Surazynski et al. (2001, 2005) monstrated that prolidase activity is enhanced by NO via increasing serine/threonine phosphorylation through PKG-cGMP pathway. This finding suggests that in absence of prolidase the inflammatory signalling pathways and regulation of the terminal step of matrix degradation and collagen turnover could be compromised, ultimately causing delay in skin repair (Surazynski et al. 2005).

In their more recent paper, Surazynski et al. (2007) correlated a delay in wound healing with a decrease in angiogenesis due to lack of prolidase through nuclear hypoxia inducible factor 1 (HIF-1α) pathway. During an inflammatory process the activation of metalloproteinases and the consequent increase in matrix degradation releases proline and hydroxyproline-containing peptides (Laitinen 1975; Rundhaug 2005). These peptides, normally degraded by prolidase, release proline and hydroxyproline, which play an important role in modulating nuclear levels of HIF-1α by inhibiting its prolylhydroxylation and decreasing its degradation (Surazynski et al. 2007). The lack of prolidase activity could cause a decrease in HIF-1α and ultimately a down regulation of the genes under its control such as Vascular Endothelial Growth Factor (VEGF) and Glucose transporter 1 (Glut-1) with a compromised angiogenic signalling.

Finally the mental retardation, from mild to very severe, frequently associated to PD, was suggested to be caused by the high amount of proline residues in neuro-peptides, whose synthesis and degradation could be compromised in absence of prolidase activity (Hui and Lajtha 1980).

Conclusion

Prolidase is a multifunctional enzyme whose biological relevance, structure and mechanism of catalysis are still only partially understood. All the hypotheses proposed to elucidate the pathophysiology of PD are useful to better understand the disorder and the biological function of prolidase, but ultimately further studies in vitro using recombinant enzyme and/or patients samples and *in vivo* animal model are necessary.

Future plans

Our laboratory is a centre for the molecular diagnosis of PD (http://www.orphanet.net) and we are continuously collecting PD patients samples to offer a valid genetic counselling and to identify new mutant alleles. We strongly believe that the characterization of new mutations will help both to clarify the relationship between function and structure and to elucidate the relationship between genotype and phenotype in this rare disorder.

In addition we are also generating a murine model for PD to develop new therapeutic trials. Although blood samples and skin biopsy followed by culture of PD cells are useful tools, the availability of a murine model for PD will be extremely valuable to perform analysis on particular tissues and in sufficient statistical number that is not possible for obvious ethical reasons using human patients.

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