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ORIGINAL ARTICLE

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Characterization of a new PEPD allele causing prolidase deficiency in two unrelated patients: natural-occurrent mutations as a tool to investigate structure–function relationship

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Abstract Prolidase deficiency (PD) is a rare autosomal recessive disorder characterized mainly by skin lesions of the legs and feet, mental retardation, and respiratory infections. Mutations at the PEPD locus, located on chromosome 19, are responsible for this disease. We identified a new PEPD allele in two unrelated Portuguese PD patients by analyses of reverse transcribed PCR-amplified cDNA. We used SSCP analysis of seven overlapping fragments spanning the entire coding region of the gene and detected abnormal SSCP bands in two of them: PD3 (nt 425–743) and PD4 (nt 661–973). Direct sequencing of the mutant cDNA and genomic DNA revealed a new homozygous 3-bp deletion (Y231del) in both cases. Transient expression in PD fibroblasts of wild-type and mutant prolidase cDNA confirmed reduced activity of the construct carrying the 3-bp deletion. The mutation results in a loss of prolidase activity in skin fibroblasts. Intracellular accumulation of Gly-Pro dipeptide in long-term cultured fibroblasts was detected by capillary electrophoresis. The mutation falls in the α2 domain of the "pita bread" structure proposed for *E. coli* and human prolidase by Bazan et al. on the bases of their sequence homology with *E. coli* methionine aminopeptidase. Taking into account the effects of the described mutations on stability and activity of the enzyme, we propose the identification of three different functional regions.

Keywords Prolidase deficiency · Prolidase · SSCP · Capillary electrophoresis

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Introduction

Prolidase (E.C. 3.4.13.9) is a ubiquitous cytosolic enzyme involved in the deactivation of neuropeptides (Hui and Lajtha 1980), in the facilitation of renal excretion of hydroxyproline and excess of proline (Jackson and Heininger 1975) and in the latter stage of both dietary and endogenous protein catabolism (Myara et al. 1984). It specifically cleaves iminodipeptides containing C-terminal prolyl or hydroxyprolyl residues thus playing an important role in the final stage of degradation of collagen, which is particularly rich in those amino acids. Prolidase is a homodimer with a subunit of Mr 54,305 and its catalytic activity requires the presence of Mn^{2+} ions (Royce and Steinmann 2002).

At the present, no crystallographic data are available for the human enzyme, but on the basis of amino acids sequence data, secondary structure predictions indicated the presence of α-helices, β-sheets, and potential β-turns with hydrophobic and hydrophilic regions distributed evenly throughout (Bazan et al. 1994). Comparison of amino acid sequences further suggests that prolidase, both from human and *E. coli* sources, are related to *E. coli* methionine aminopeptidase (AMPM, E.C. 3.4.11.18) whose tertiary structure comprise a so called "pita-bread" fold with two bivalent metal ion binding regions within the active site (Bazan et al. 1994; Roderick and Matthews 1993).

The prolidase gene (Peptidase D, PEPD) is located on chromosome 19 (19p12–p13.2) and contains 15 short exons spanning over 130 kb of genome (Tanoue et al. 1990). Its transcript is a 2.3 kb mRNA that in humans

encodes a polypeptide of 492 amino acids (Endo et al. 1989). The loss of prolidase activity is responsible for prolidase deficiency (PD), a rare autosomal recessive inherited disorder with an estimated incidence of one to two cases per 1 million births (Royce and Steinmann 2002). The dominant clinical manifestation associated with the disorder is intractable skin ulcerations particularly on the legs and feet. The development of ulcers is often preceded by other dermatological manifestations such as erythematous papular eruptions, telangiectasia with pruritus and photosensibility, impetigo-like eruptions, pruritic eczematous lesions, and necrotic papules. The phenotype is extremely variable and typically includes different levels of mental retardation, recurrent respiratory infections, splenomegaly, and dysmorphic facies. The age of onset of PD varies from birth to 22 years; a case of late onset had also been described (Dyne et al. 2001). A few prolidase-deficient individuals have been reported as asymptomatic, but, given time, the great majority of such individuals developed symptoms of greater or lesser severity (Royce and Steinmann 2002). Any correlation between the clinical manifestations of PD and the putative roles of the enzyme is still difficult to discern, indicating that the physiological functions of the enzyme are still not completely understood.

At present, only five point mutations—one triplet deletion, four splicing errors, and a large deletion in the prolidase gene—have been reported as causative for the disorder (Royce and Steinmann 2002). We believe that the identification of new mutations is a valuable tool both for better understanding the relation between function and structure of the enzyme and to elucidate the relationship between molecular defects and clinical outcome. In this study, we describe the molecular and biochemical characterization of a novel PEPD allele identified in two unrelated PD cases, and we attempt to delineate a map of causative mutations in relation to the structure and function of the enzyme.

Materials and methods

Cell strains and culture conditions

Fibroblasts from patients P1 and P2 (T745 and T410, respectively) were obtained by one of the coauthors, Dr Vilarinho (Instituto de Genetica Medica, Hospital Criancas Maria Pia, Porto, Portugal). Control fibroblasts were purchased from International Pbi SpA (Milan, Italy). The patients' clinical phenotype has been previously reported (Pereira and Vilarinho 1997; Lopes et al. 2002). Cells were grown at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Sigma, Milan, Italy) supplemented with 10% fetal calf serum (FCS, Euroclone, Pero, Milan, Italy) or 1% ITS + 3 serum substitute (Sigma, Milan, Italy) that does not contain prolidase enzyme. Cells were used between the fourth and tenth passages.

Reverse transcription–PCR amplification and SSCP analysis

Total cellular RNA was isolated from cultured fibroblasts using TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA synthesis was carried out with first strand cDNA synthesis kit for RT-PCR (Roche, Monza, Milan, Italy) according to the manufacturer's specifications. The entire prolidase transcript was amplified as seven overlapping fragments approximately 300–400 bp long using Taq polymerase (Clontech, San Jose, CA, USA) and the primer pairs and PCR conditions described by Forlino et al. (2002). Both for patients and controls, all amplified fragments were first run on 1.8% agarose gel and then analyzed by single-strand conformation polymorphisms (SSCP). Aliquots of the PCR products were denatured at 95°C for 5 min and placed on ice prior to electrophoretic separation. Horizontal ultrathin gel (0.3 mm) composed of 15% acrylamide (acrylamide/piperazine diacrylamide, 85:1) with 8% glycerol in Tris–formate buffer (120 mM, pH 9.0) were cast by the flap method on gel-bond films (FMC, Rockland, MN, USA). Tops and bottoms of the gels were covered with paper wicks soaked in 2×TBE buffer, and the gels were run in a Pharmacia Multiphor II electrophoresis apparatus (Ambion, austin, TX, USA) at 14°C for about 90 min at a constant 12 W. Bands were visualized by silver staining (Superti-Furga et al. 1996).

Sequencing

Overlapping fragments PD3 (nt 425–743) and PD4 (nt 661–973) for both patients and controls were gel-purified and directly sequenced by the dideoxy chain termination method (Sanger et al. 1977) using the ABI prism bigdye terminator cycle sequencing ready reaction kit (Perkin Elmer, Boston, MA, USA). Primers were the same as used for the amplification reactions (Forlino et al. 2002).

Northern blotting analysis

Total cellular RNA from control and patient cultured fibroblasts were separated on denaturing 1% agarose gel, blotted onto a nylon membrane (N+-Hybond, Amersham, Biosciences Europe GmbH, Cologno Monzese, Milan, Italy), and hybridized to a prolidase cDNA probe (PD1s–PD2as; −30/487 nt). Hybridization was performed at 42°C overnight using UltraHyb hybridization buffer (Ambion, Austin, TX, USA) according to the manufacturer's suggestion. The probe was radiolabelled with α^{32}P dCTP (3,000 Ci/mmol) using ready-to-go labeling kit (Amersham Biosciences Europe GmbH, Cologno Monzese, Milan, Italy). Equal loading of the samples were verified by hybridization of

the membrane with radiolabelled 18S probe (Ambion, Austin, TX, USA).

Genomic DNA amplification

Genomic DNA was extracted from cultured skin fibroblasts (Maniatis 1982). About 0.5 μg of DNA was amplified by PCR using as sense primer 5′CACTGACTGCCAGGTCGAGTGGCT 3′ (nt 110917–110940, intron 9, clone AC008744) and as antisense primer 5′CTCCTACACCTGCATCTGCGGCAG 3′ (nt 733–756). PCR products were run on 1.5% agarose gel, gel purified, and directly sequenced with the same primers used for the amplification.

Prolidase assay

Patient and control fibroblasts were grown to confluence in T75 tissue culture flasks in DMEM supplemented with 10% FCS. Prolidase activity was determined according to the procedure of Myara et al. (1982), which is based on the measurement of free proline by Chinard's reagent (Chinard 1952). Protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Segrate, Milan, Italy) using bovine serum albumin as standard.

Plasmid construction

Total cellular RNA, isolated from cultured human control fibroblasts, was extracted by TriReagent (Molecular Research Center, Inc., Cincinnati, OH, USA), 1 μg was reverse transcribed using the Gene Amp Gold RNA PCR kit according to the manufacturer's protocol. Primers 5′-AGGTACCTATGGCGGCGGCCACCGGACCCT-3′ (nt 17–38) and 5′-GATCTAGATGATTCTGGGTGCCGTCTCTCGCTAC-3′ (nt 1582–1601) sense and reverse, respectively, carrying 5′ overhang sequences containing restriction sites (XbaI and KpnI) immediately adjacent to the cDNA were used to amplify nucleotides 1–1584 of the prolidase cDNA by PCR. The amplified product was subcloned into the eukaryotic-expressing plasmid pcDNA4/HisMax-A (Invitrogen, Milan, Italy). This vector contains the strong CMV promoter, a SP163 enhancer, the sequence coding for a poly-histidine tag, and the sequence for enterokinase recognition site. QuickChange II XL site-directed mutagenesis kit (Stratagene) was used according to the instructions to create the mutant expression vector containing the Y231 deletion. Complementary mutagenic primers 33 bp long, 5′- CTTCGAGCACTACTGCTCCCGGGGCGGCATGCG-3′ and 5′-CG-CATGCCGCCCCGGGAGCAGTAGTGCTCGAAG-3′ (nt 691–726) were used to introduce the specific delTAC707–709. The nucleotide sequence of the wild-type and mutant constructs was confirmed by sequencing.

Transfection

Fibroblast cells from patient P1 were plated at 3×10^5 cell density in 60 mm petri dishes and transfected, respectively, with no plasmid or 8 μg of wild-type or mutant construct. Lipofectamine 2000 Reagent (Invitrogen, Milan, Italy) was used for transfection following the manufacturer's suggestion. Two micrograms of the plasmid pcDNA4/HisMax-LacZ, which harbors the β-galactosidase gene of *E. coli*, was cotransfected to standardize for transfection efficiency. Cells were harvested 48 h posttransfection. Cells were resuspended in 50 mM Tris pH 7.8, and lysates were obtained by three rounds of freezing and thawing. Protein concentration was measured by BCA assay (Pierce). β-Galactosidase activity was assayed by the β-Gal assay kit (Invitrogen, Milan, Italy) following the manufacturer's suggestions. Cell extracts were used for prolidase assay as described previously.

Western analysis

Twenty micrograms of protein from transfected cell lysates were separated by 8% SDS-PAGE and transferred to nylon membrane (Hybond-N, Amersham). Prolidase was detected by using 1:5,000 dilution of anti-HisG antibody (Invitrogen, Milan, Italy) and the ECL Plus (Amersham) according to the manufacturer's protocol.

Imidodipeptide identification and quantitation

Imidodipeptide identification and quantitation were performed as described by Lupi et al. (2003). Briefly, 8×10^5 patient and control fibroblasts were plated in T75 flasks and grown for 8 days in DMEM supplemented with 1% ITS + 3 serum substitute. Upon medium removal and three PBS washes, the cell layer fractions were dissolved in 2 ml of 1% (w/v) Triton X-100, 0.6 M KCl in PBS by shaking for 30 min at room temperature. The samples were denatured at 80°C for 15 min, and protein determination was performed using Bio-Rad protein assay. For capillary electrophoresis analysis, a Beckman (Palo Alto, CA, USA) P/ACE 2100 instrument was used. An untreated fused-silica capillary of 50 μm i.d. and 50 cm effective length was adopted. Analyses were performed by capillary zone electrophoresis (CZE) using 50 mM sodium tetraborate buffer pH 9.3 containing 30 mM α-cyclodextrine. Samples were injected for 60 s, and a 30 kV current was applied. The Beckman P/ACE Station software was used for data analysis, and results are reported as mean ± SD of $n=3$; they were analyzed by t test. $P<0.05$ was considered statistically significant. Gly-Pro peak identification was performed by coinjection of the samples with commercially available standard (Sigma, Milan, Italy).

Results

SSCP and mutational analysis

SSCP analysis revealed aberrant band patterns indicative of mutation for both patients in the overlapping fragments PD3 and PD4 suggesting the presence of a molecular defect between nt 661 and nt 743. No electrophoretic mobility shifts were observed for any of the other RT-PCR products (data not shown). Direct sequencing of the RT-PCR products revealed that both patients carried in exon 10 an in-frame 3-bp (707–709delTAC) deletion that results in the removal of the residue Y231 (Fig. 1a,b). Neither SSCP analysis nor direct sequencing identified a second mRNA species in these patients. Amplification and sequencing of genomic DNA showed homozygosity for the 3 bp deletion (Fig. 1c,d).

Prolidase expression and activity of control and proband cultured fibroblasts

Northern blot analysis of total RNA from P1 and P2 and control, using prolidase specific probe and 18S probe as internal standard, showed the presence of a normal amount of the 2.3 kb mRNA (Fig. 2).

In the cultured dermal fibroblasts taken from the two PD patients, we found a strong reduction in catalytic activity of the prolidase enzyme to 5% of control value. This data is in agreement with results previously reported for the two probands (Pereira and Vilarinho 1997; Lopes et al. 2002).

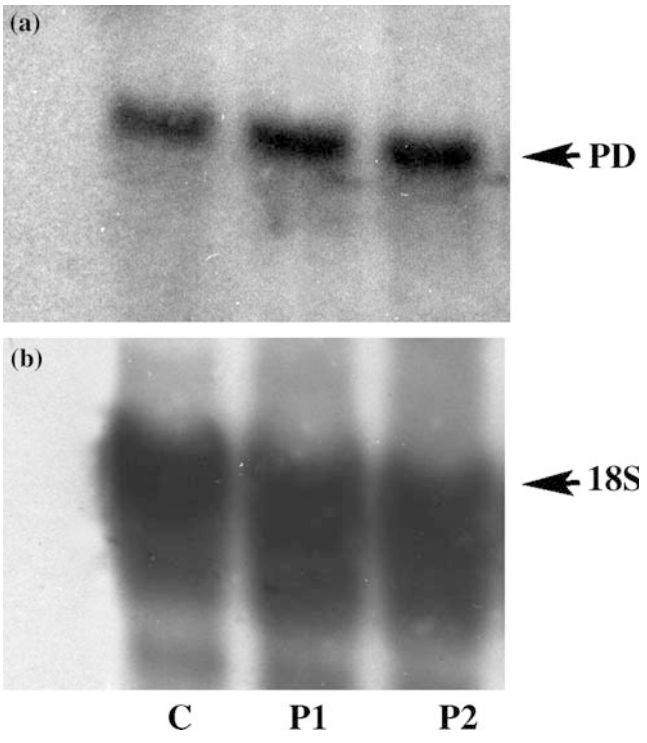

Fig. 2a, b Northern blot analysis of RNA obtained from cultured fibroblasts. **a** A prolidase-labeled probe was used in hybridization. **b** Equal loading of the lanes was evaluated by hybridization of the same membrane with an 18S-labelled probe

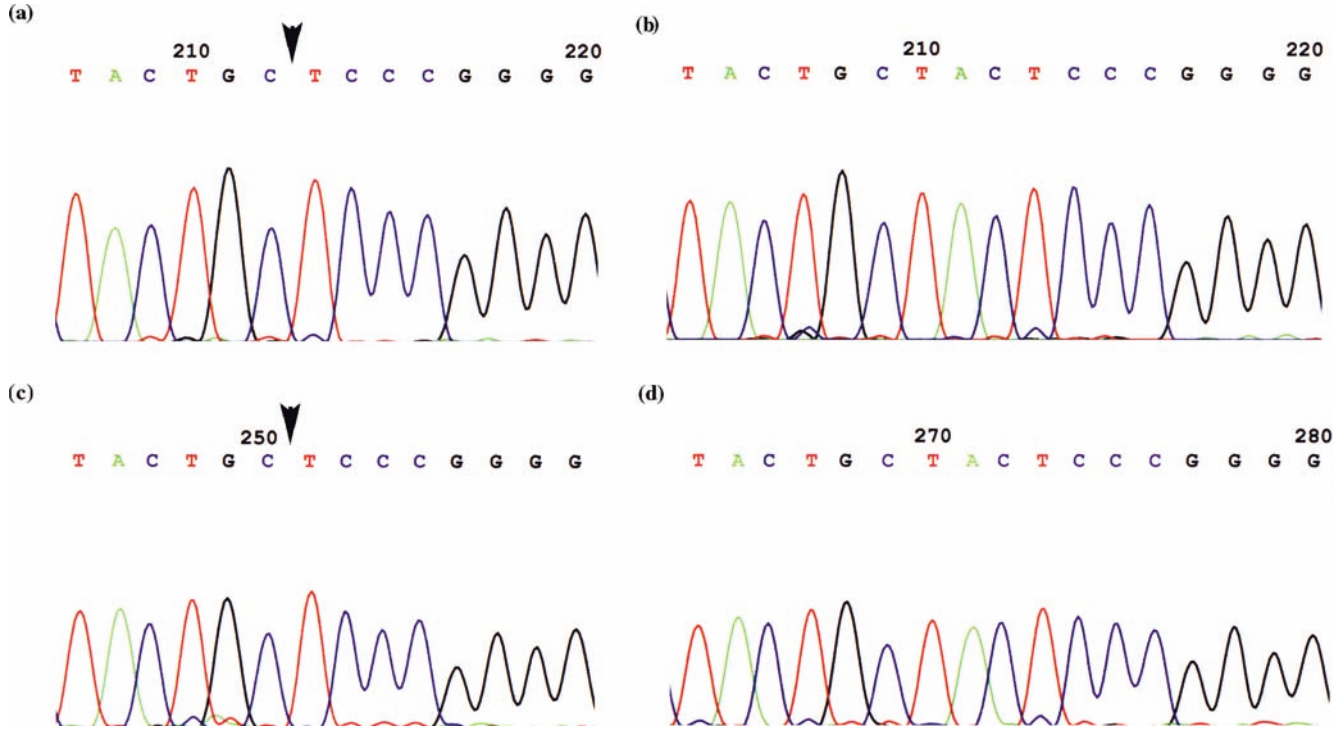

Fig. 1 **a**, **b** Direct sequencing of cDNA; and **c**, **d** genomic DNA. Electropherograms showing, respectively, **a**, **c**: 707–709delTAC mutation causing the Y231del in patients 1 and 2; **b**, **d**: the control sequence in the same region

Iminodipeptide quantitation in cultured fibroblasts

Among the other dipeptides, we decided to measure the Gly-Pro content in the cell layer of proband- and control-cultured fibroblasts, since these cells, used for the experiments, synthesize a large amount of type I collagen, which is rich in these amino acids. The amount of Gly-Pro in the cellular extract obtained from 8-day-cultured fibroblasts from patients and control was evaluated by capillary electrophoresis. The peak was identified by coinjection with commercially available standard. The peak area, expressed in arbitrary units, was converted in nanomoles of Gly-Pro by means of an external calibration curve obtained by injecting a known amount of standard dipeptide in the same buffer used to solubilize the samples. A significant increase of Gly-Pro was detected in both patients with respect to the control (Table 1).

Prolidase expression in fibroblast cells

The effect of the 707–709delTAC on prolidase activity was measured by expression analysis in PD-cultured fibroblasts. P1 cells were transfected using wild-type and mutant prolidase vector (Y231del). Prolidase activity was normalized for transfection efficiency. The mutant recombinant enzyme had a markedly reduced activity: 10% of the wild-type recombinant enzyme (Fig. 3a).

A protein of the expected size (about 56 kDa) cross-reactive with the anti-HisG antibody was present in cells transfected with mutant and wild-type vectors, although the Y231del prolidase appeared to be unstable (Fig. 3b). The protein level of the mutant recombinant prolidase was 37% of the recombinant wild-type enzyme.

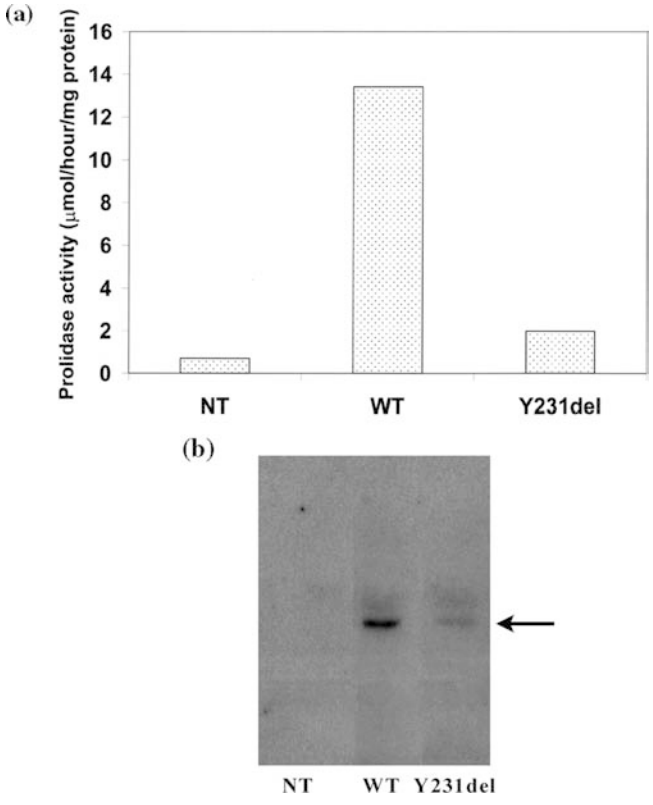

Fig. 3 **a** Expression of wild-type and mutant PEPD alleles in P1 human fibroblast cells. Enzyme activity of P1 cells not transfected (NT), transfected with the wild-type prolidase construct (WT), and transfected with the mutant prolidase construct (Y231del) is shown. **b** Western blot analysis of P1 fibroblast cells expressing the recombinant human prolidase. An antibody directed against the His Tag epitope was used. Twenty micrograms of proteins from P1 human fibroblasts not transfected (NT), transfected with the wild-type prolidase construct (WT), and transfected with the mutant prolidase construct (Y231del) were loaded on 8% polyacrylamide gel

Discussion

Molecular analysis showed that the two unrelated Portuguese patients had the same homozygous 3-bp deletion in exon 10 of the prolidase gene. The expression level of the mutant transcript was similar to the control, but the activity of the mutant enzyme was reduced to 5% of the control value in cultured skin fibroblasts. Expression analysis of mutant recombinant prolidase revealed a markedly reduced activity (10% of wild-type) and protein stability (37% of wild-type). Based on searches of protein data banks using sequence and structure-based profiles, Bazan et al. had previously proposed a strong homology between the *E. coli* and human prolidase and the *E. coli* methionine aminopeptidase (AMPM) sharing the same "pita-bread" fold (Bazan et al. 1994). According to this homology, deletion of the Y231 would be located in the $\alpha 2$ region of the "pita-bread" fold, and its absence could affect the structure and function of the enzyme.

Table 1 Intracellular Gly-Pro content in control and patients cultured fibroblasts

Cases	nmol Gly-Pro/mg proteins ± SD
Control	15.84 ± 0.95
P1	22.53 ± 0.69
P2	18.69 ± 2.13

A detailed analysis of mutations previously identified as causative for PD revealed that the ones falling in the "pita bread" fold are in agreement with the homology model proposed by Bazan et al. (1994) and with the prolidase active site proposed by Mock and Liu (1995). These molecular defects in fact compromise the function of the enzyme, either affecting its stability or its activity. Mutations located elsewhere delineate other possible domains along the prolidase sequence. A careful observation of the complete mutation map (Fig. 4) allows the distinction of three domains: (1) an N-terminal region, from exon 1 to exon 4, where no mutations had been identified, and that probably is not relevant either for stability or activity of the enzyme; (2) a central region,

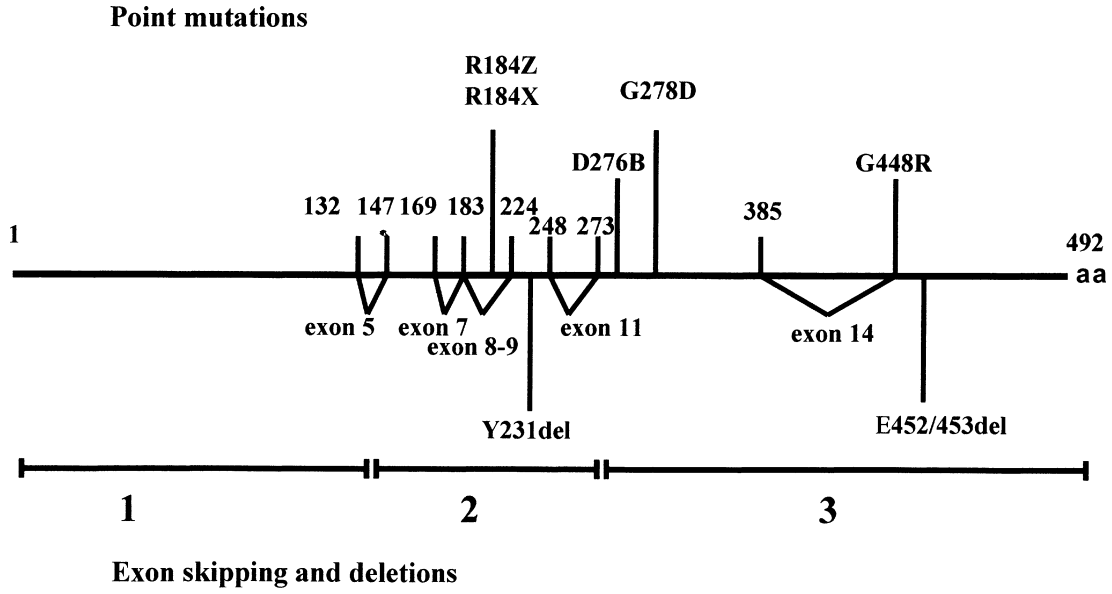

Fig. 4 Map of known mutations in the PEPD locus as causative for prolidase deficiency: point mutations on *top* are indicated with the amino acid position at which they occur and the type of residue substituted. Splicing errors and deletions on the *bottom* are indicated by the first and last amino acid residues of the skipped exons or with the position of the skipped amino acid. Three regions indicated as 1, 2, 3 on the *bottom* of the figure had been identified to have different relevance in the prolidase enzyme

from exon 5 to exon 11, that seems to be relevant for stability either of the transcript and/or of the protein; and (3) a C-terminal region, from D276 to the end of the sequence, where the active site will be located, which is important for enzyme activity (Fig. 4).

In details, the in-frame deletions of exon 5 and exon 7 (Ohhashi et al. 1990), located out of the "pita-bread" structure, respectively, cause the loss of 15 and 16 amino acids and the synthesis of either an unstable transcript or an unstable protein; the deletion of exons 8–9, falling in the α1 and α2 region of the "pita-bread" structure, compromised the stability of the transcript (Forlino et al. 2002). Two different mutations at position 184 had been described: R184Z and R184X (Kikuchi et al. 2000; Ledoux et al. 1996). Position 184 is located in a non-conserved region of the protein, but it is a well-conserved amino acid (Maher et al. 2004) distal to the hypothetical active site although just one position 5′ to the α1 region of the "pita bread" fold. It would seem that nonsense mutation (STOP) affects stability of the catalytically inactive enzyme due to the synthesis of a short transcript, whereas the missense mutation does not affect stability and allows production of a partially active enzyme.

The skipping of exon 11 causes the deletion of the β_A and β_B domains according to Bazan et al. model and results in unstable transcript and inactive enzymes (Forlino et al. 2002). The deletion of the sequence of a full exon in region two of the prolidase compromises enzyme stability. Previously reported data demonstrated that the abundance and stability of mRNA generated by alternative sites depends on whether the products are inframe, whether they lead to premature termination codons within the coding sequence, and whether the termination codon is succeeded by an intron (Carter et al. 1996; Maquat 1996; Frischmeyer and Dietz 1999). Furthermore, the existence of an mRNA surveillance system, which degrades aberrant mRNA in eukaryotic cells by recognizing the spatial relationship between the termination codon and specific downstream sequence information, has been demonstrated (Hilleren and Parker 1999).

The first mutation to be identified in two unrelated PD patients resulted in a D276N change (Tanoue et al. 1991b) located in the β_B region. This residue corresponds to D97 in *E. coli* AMPM, a cobalt ligand in the active site. Point mutation 826G > A causing the G278D also is located in the β_B region, and 1342G > A causing the G448R both introduce a charged residue in close proximity to the negatively charged active site metal-binding residues D276 and E452 (Ohhashi et al. 1990; Ledoux et al. 1996). Ledoux et al. speculate that the function of these metal-binding residues might be affected by steric or electronic hindrance as a result of these mutations with consequent loss of enzyme activity. The E452/453del removes one of the metal binding site (Ohhashi et al. 1990). The deletion of -774 bp that eliminates all exon 14 causes the loss of β'_B and β'_C regions according to the model of Bazan et al. and also includes the loss of G448. The protein was synthesized as shorter polypeptide of Mr 49,000 but resulted to be inactive (Tanoue et al. 1990, 1991a).

We finally performed the analysis of intracellular Gly-Pro in long term-cultured fibroblasts by capillary electrophoresis. In our two patients, as already demonstrated for five other PD cases (Forlino et al. 2002), there was a statistically significant accumulation of dipeptide in the cellular extract with respect to the control cells

(Table 1). This observation suggests that Gly-Pro retention could be a common feature in PD cells and might be linked to the phenotypic expression of the disease, although further study needs to be performed. In fact, due to the limited number of patients analyzed, it is still difficult to draw a link between the amount of intracellular Gly-Pro and the severity of the outcome. The characterization of a new PD mutant allele and the development of an eukaryotic expression system for mutant human prolidase will provide useful tools to study the function–structure relationship of the enzyme.

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