Intestinal lactase-phlorizin hydrolase (LPH): the two catalytic sites; the role of the pancreas in pro-LPH maturation

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Abstract Brush border lactase-phlorizin hydrolase carries two catalytic sites. In the human enzyme lactase comprises Glu-1749, phlorizin hydrolase Glu-1273.
The proteolytic processing of pro-lactase-phlorizin hydrolase by (rat) enterocytes stops two amino acid residues short of the N-terminus of 'mature' final, brush border lactase-phlorizin hydrolase. Only these two amino acid residues are removed by luminal pancreatic protease(s), probably trypsin.

Key words: Lactase; Active site; Proteolytic processing; Pancreas

1. Introduction

In the biology of lactase-phlorizin hydrolase (LPH, which is an intrinsic protein of the small-intestinal brush border membrane and splits lactose, cellobiose, glycosyl-β-ceramides and a number of aryl-β-glycosides including phlorizin and *o*- and *m*-nitro-phenyl-β-glycosides), at least two issues have been the objects of contrasting reports: (i) is the 'phlorizin hydrolase' catalytic site identical with the 'lactase' site? and (ii) in the multistep proteolytic processing of pro-lactase-phlorizin hydrolase (pro-LPH) to 'mature', brush border LPH what is the precise role (if any) of luminal, pancreatic proteases, in particular of trypsin? In the present paper we provide what we believe are the answers to these only partially related questions.

2. Materials and methods

2.1. Mutagenesis and construction of cDNA clones

Human pre-pro-LPH cDNA was subcloned from the plasmid pSCTmLPH (human pre-pro-LPH [1]) into the vector pGEM-2 (Promega, Switzerland) and the resulting plasmid was used for site-directed mutagenesis using the Unique Site Elimination mutagenesis kit (Pharmacia, Switzerland). For pre-pro-LPH E1273G the sense primer 5′-TAC ATC ACC GGA AAC GGA GTG-3′ was used and for pre-pro-LPH E1749G the primer 5′-TAT GTC ACC GGA AAT GGA GTG-3′. The double mutant was constructed by using both primers simultaneously. The mutations were confirmed by DNA sequencing. The mutated and wt LPH cDNA was excised with *Eco*RI and cloned into the *Eco*RI site of pcDNA I/Amp (Invitrogen, Switzerland).

2.2. Transient transfection of cells

COS-7 cells, cultured in DMEM/10% FCS (Life Technologies, Switzerland), were transiently transfected as described by Chen and Okayama [2]. One day before transfection, the cells were seeded into 6-well plates, to reach approximately 80% confluence the next day. During the last 30 min prior to transfection, the cells were incubated in fresh 1.5 ml DMEM/10% FCS at 37°C and 3.5% CO_2. The calcium phosphate-DNA mixture was prepared by mixing 5 μg of plasmid DNA with 100 μl of 250 mM $CaCl_2$ and 100 μl of 2×BBS (50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH adjusted with NaOH to exactly 6.95) and incubating for 15 min at room temperature. 150 μl of this mixture were then slowly added to the cells, and incubation was continued for 24 h at 37°C and 3.5% CO_2. The following day, the cells were washed twice with phosphate buffered saline (PBS), refed 2 ml of fresh medium, and incubated for 24 h at 37°C and 5% CO_2.

2.3. Enzyme assays and protein determination

COS-7 cells transfected with LPH wt or LPH mutants were detached with a rubber policeman and collected in cold PBS. The cells were disrupted with a conical grinding pestle and sonicated (15 s, 60 W). Lactase activity was determined as described by Wacker et al. [3]. Briefly, cell homogenates were incubated at 37°C for 1 h in 33 mM sodium maleate buffer, pH 6.0 using 33 mM lactose as substrate. After boiling for 2 min the liberated D-glucose was determined at 365 nm with D-glucose dehydrogenase. Phlorizin hydrolase was determined using 3 mM phlorizin as the substrate in 33 mM sodium maleate buffer. After incubation at 37°C for 4 h the reaction was stopped by boiling and the liberated D-glucose was determined as described above. Protein concentrations were determined by the method of Bradford [4].

2.4. Animals, surgical procedures and sequence determination

All manipulations were performed as described [5]. Briefly, adult male Wistar rats were anaesthetised by ether inhalation. A median laparotomy was performed. The jejunal resection started 3 cm downstream of the Treitz ligament toward the ileum (∼30 cm). Intestinal continuity was re-established by an end-to-end anastomosis. The jejunum was exteriorised as a Thiry-Vella loop with two stomata located on the abdomen. Control animals underwent intestinal transection without removal of tissue, followed by re-anastomosis (sham operated). After animals were killed (15 to 40 days after operation), the isolated Thiry-Vella loop and the anastomoses between the jejunum and the ileum (upstream and downstream of the Thiry-Vella loop, respectively) were removed. In sham-operated animals the part of the jejunum corresponding to the isolated loop was used. Mucosa was removed and brush border membrane vesicles prepared by the magnesium precipitation procedure [6]. Rat LPH was immunoprecipitated from brush border vesicles containing 250 mg of protein using a polyclonal guinea pig anti-rabbit LPH antibody [7], as described by Lottaz et al. [8], resolved by SDS-PAGE, transferred to a PVDF membrane and subjected to N-terminal sequence analysis on an Ap-

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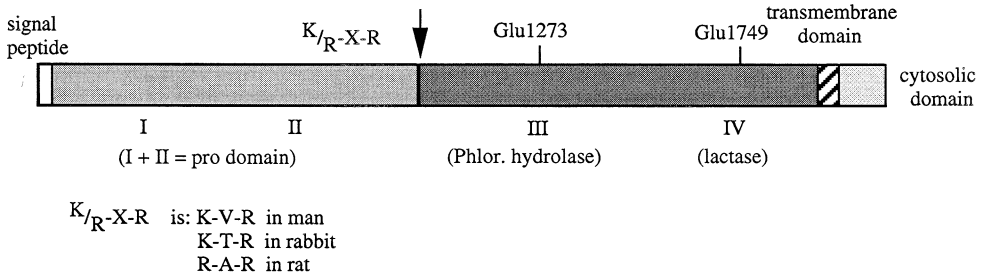

Fig. 1. Schematic representation of pre-pro-LPH. Individual regions of the enzyme are indicated by the differently shaded bars. The roman numbers underneath the LPH scheme refer to the four homology regions. The vertical arrow marks the beginning of mature brush border lactase. Amino acid numbering refers to the respective position in the human enzyme.

plied Biosystem 476A protein sequencer equipped with a ProBlott cartridge.

3. Results and discussion

3.1. The number and location of the hydrolytic sites in mature, brush border LPH

A number of experimental approaches have shown that lactase, as isolated from the brush border membrane, carries two catalytic sites, i.e. one splitting i.a. lactose, cellobiose [9,10], *o*-nitro-phenyl-β-glucopyranoside and -β-galactopyranoside (e.g. [11]), and the other splitting i.a. phlorizin, β-glycopyranosyl-ceramides [12], *m*-nitro-phenyl-β-glucopyranoside [3,11]. The two sites are differently inactivated by heat [11–15], and by an active site-directed covalent inhibitor [3]. Two moles of the inhibitor are incorporated per mol of rabbit LPH [3] at positions Glu-1271 and Glu-1747, i.e. within the homologous regions III and IV, respectively (see Fig. 1). The two active sites show little or no inhibition between the respective substrates [13–15]. Yet, others [16], have recently attributed both lactase and 'phlorizin hydrolase' activities almost completely to a single site (at Glu-1750, in the rat enzyme). However, the substrate used by these authors was *ortho*-nitro-phenyl-β-glucopyranoside, which is an excellent substrate for lactase, but is only marginally split by the phlorizin hydrolase site [11,15]. It is the *meta* isomer that is preferentially split by the latter [3,11].

We have thus examined by site-directed mutagenesis (as in [16]) the location and function of the critical Glu residues in human LPH, but using lactose and phlorizin as the representative substrates. To localise the two active sites in LPH produced in COS-7 cells the mutants E1273G and E1749G were prepared. COS-7 cells were transiently transfected and incubated for 48 h before cell homogenates were prepared for determination of lactase and phlorizin hydrolase activity, respectively[3]. As summarised in Table 1, mutation of E1273 to G maintains lactase activity, but reduces phlorizin hydrolase activity to about 10%. Conversely, mutation of E1749 to G completely abolishes lactase activity, but maintains 75% phlorizin hydrolase activity. Simultaneous mutation of both active site E residues removes both enzymatic activities. The targeting of all these mutants at the plasma membrane was verified by cell surface immunofluorescence (data not shown), demonstrating that the introduced mutation did not influence enzyme transport. From these data it is clear that: (i) LPH does carry two catalytic sites which can be site mutated independently of one another; and (ii) the phlorizin hydrolase site comprises Glu-1273, whereas the lactase site (as reported by [16] also) comprises Glu-1749. From chemical labelling and peptide degradation we originally attributed Glu-1271 to lactase and Glu-1747 to phlorizin hydrolase in the rabbit [3]. It is likely that this discrepancy is due to the difficulty of attributing unequivocally to one or the other site highly homologous glycosylated peptides.

Lactase is thus the site closer to the brush border membrane (the membrane-spanning stretch is between positions 1883 and 1901, [17], see Fig. 1). These results are not in disagreement with the recent report that expression of *both* region III and IV is necessary for the appearance of either activity: the expression of region III or of region IV alone does not lead to enzymatically active products, not even in duplication (i.e. two regions III or two regions IV) [18]. Some sort of interaction between regions III and IV may well take place within mature LPH for the appearance of their catalytic activities.

3.2. Pancreatic juice and the last proteolytic step(s) in the processing of pro-LPH to mature, brush border LPH

Small-intestinal LPH is synthesised as a single-chain precursor of more than 1920 amino acid residues in length (1927 in man; 1926 in the rabbit [17]; 1928 in the rat [19]). The leading sequence is split in the ER at the site predicted on the basis of von Heijne's algorithm, i.e. after G19 in the human enzyme (after G21 in the rat enzyme). The resulting pro-LPH undergoes, in addition to the usual glycosylations, proteolytic processing events, which lead to mature brush border LPH. In vitro human pro-LPH can be split by trypsin to LPH (or to a 'mature-like' LPH) [20] and indeed some [21] have even suggested that in vivo also, luminal trypsin may play a major role in processing pro-LPH to mature lactase.

However, a number of studies using in vitro primary cultures of small intestine have shown that most if not all the proteolytic processing of pro-LPH to LPH (or to a 'mature-like' form) takes place wholy or mostly within the enterocytes. The complete removal of the pro-sequence, i.e. of regions I and II (Fig. 1) involves more than a single proteolytic event and intermediates are formed, which differ in the various species, due to differences in the amino acid sequences and/or to different repertoires in pro-protein convertases. For example,

[3] As shown by the very low phlorizin hydrolase activity in the mutant where the catalytic E in LPH had been mutated to G (Table 1), only a small fraction of this activity in COS cell homogenates is not associated with LPH. Hence in addition to lactase, for this activity also it was legitimate to use total homogenates. However, in cell types other than COS (e.g. in small intestine) the contribution of other β-glycosidases (typically, lysosomal ones) to total phlorizin hydrolase may be significant.

Table 1
Lactase and phlorizin hydrolase activity of wild-type and mutant enzymes generated in COS-7 cells

	Wild-type	Mutant E1273G (domain III)	Mutant E1749G (domain IV)	Mutant E1273G/E1749G (domains III+IV)
Lactase	100%	88 ± 6%	−3 ± 2%	−1 ± 1.5%
Phlorizin hydrolase	100%	9 ± 7%	76 ± 13%	13 ± 5%

The activities of wild-type enzymes are set to 100%. Data are expressed as means ± S.E.M. of one selected experiment done in triplicate.

in the rabbit a 180 kDa intermediate is formed because in this species (but not in man) a furin consensus sequence occurs in the second half of region I [22,23], whereas in humans (in vitro cultured peroral biopsies) the intermediate is a 'mature-like' LPH which is only 4 kDa larger than 'mature' LPH and which can be trimmed to the size of LPH by adding trypsin in vitro [24,25].

We have recently shown that mature or 'mature-like' LPH prepared from the rat intestinal tract which has not been exposed to pancreatic proteases (i.e. Thiry-Vella loops [5]), had an apparent molecular mass of 130 to 140 kDa, i.e. was nearly indistinguishable from that of mature LPH, suggesting the existence of a further cleavage step very close to the native N-terminus. In order to identify this site, LPH has now been immunopurified from normal, from sham-operated rat intestine, from the jejunum-ileum anastomoses of operated animals, as well as from their Thiry-Vella loops, resolved by SDS-PAGE, transferred to a PVDF membrane and subjected to N-terminal amino acid sequence analysis. As shown in Table 2, rat lactases which had been exposed in vivo to pancreatic juice started with V870: Identical N-terminal sequences were obtained from normal rats, from sham-operated animals or from the jejunum-ileum anastomosis, all of which were exposed to pancreatic proteases. Contrary to this, 'mature-like' rat LPH from the Thiry-Vella loops was two amino acids longer and began at A868. This result showed that in the rat the lactase forms were in fact subjected to nearly complete proteolytic conversion to mature LPH by enterocytic proteases before finally loosing the last two extra amino acids through the action of pancreatic protease(s), most likely trypsin. It is noteworthy that the sucrase-isomaltase from the Thiry-Vella loops was in the form of unsplit pro-sucrase-isomaltase (see [5]), as it always is when synthesised in vivo or in vitro in the absence of pancreatic juice (reviewed in [26]), thereby providing an internal control.

Most of the proteolytic processing of pro-LPH takes place intracellularly ([24,25], J.E. Mesonero, S.M. Gloor and G. Semenza, in preparation). It has been shown recently (Mesonero et al., in preparation) using long chase times, that PCs are involved; this had not been observed in previous work from various laboratories, including ours, in which short chase times were used. The identified cleavage sites are some kDa upstream of the N-terminus of final 'mature' LPH ([24,25], Mesonero et al., in preparation); on the other hand, as we show here (Table 2), trypsin in vivo removes only the two amino acid residues immediately upstream of the final N-terminus. It is not obvious which enterocytic protease(s) trim the partially processed pro-LPH to position −2 (i.e. before trypsin 'takes over'). Residue R867 is not a part of a furin-like consensus sequence (there are a number of them further upstream). One could envisage lysosomal cathepsin(s) (e.g. cathepsin B), because pro-LPH has been suggested to be exposed to lysosomal proteases [27] (perhaps en route to the brush border membrane?). Another likely candidate could be granzyme A which is known to split after a Lys or an Arg residue [28]. Alternatively, the intermediate lactase forms LPH, arising from the action of furin-like PC(s) on pro-LPH, could be trimmed by brush border aminopeptidases on their way from the Golgi to the brush borders, and/or even in the latter.

Whatever the proteases involved, they are likely to act in this segment of the pro-LPH sequence on the rabbit and the human enzymes also, because of the extensive homology among the three species in the sequences immediately preceding the N-terminus of mature LPHs (Table 2). Unfortunately, we failed to obtain Thiry-Vella loops in rabbits and had no access to an equivalent small intestine from humans.

In conclusion, pancreatic juice plays a secondary role in the processing of pro-LPH to LPH, both qualitatively as shown here (only two amino acids are normally trimmed away) and quantitatively (some high molecular weight lactases escape the intra-enterocytic processing and are found in the brush border membrane [5] where they may be converted to LPH by luminal trypsin). We have thus provided now further molecular details to the concept that pancreatic juice plays a minor role in the processing of pro-lactase (Alpers and Tedesco [29]).

Table 2
N-terminal amino acid sequence of mature rat LPH either exposed (mature brush border LPH) or not (LPH from Thiry-Vella loop) to pancreatic proteases and partial amino acid sequences in the pertinent regions of pro-LPH of man, rabbit and rat

LPH	N-terminal amino sequence
Human pro-LPH	…T V N L P S K V R868 A F T F P S…
Rabbit pro-LPH	…S A H L P S K T R866 A S A L P S…
Rat pro-LPH	…R A D F T S R A R869 V T D S L P…
Mature brush border rat LPH[a]	V T D S L P…
Rat LPH from Thiry-Vella loop	X R869 V X D X…

[a]LPHs isolated from normal, or sham-operated rats, or from the jejunum-ileum anastomosis (which is exposed to pancreatic juice) of Thiry-Vella loop carrying rats yielded all the same N-terminal sequences.

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