The Prosequence of Human Lactase-Phlorizin Hydrolase Modulates the Folding of the Mature Enzyme*

Ralf Jacob, Karen Peters, and Hassan Y. Naim†

From the Department of Physiological Chemistry, School of Veterinary Medicine Hannover, Bünteweg 17, Hannover D-30559, Germany

Received for publication, December 3, 2001, and in revised form, December 10, 2001
Published, JBC Papers in Press, December 18, 2001, DOI 10.1074/jbc.M111500200

The efficient transport of proteins along the secretory pathway requires that the propeptide adopts a stably folded conformation to egress the endoplasmic reticulum (ER). The transport-competent precursor of the brush border enzyme LPH, pro-LPH, undergoes an intracellular cleavage process in the trans-Golgi network between Arg734 and Leu735 to yield LPHβinitial. The role of the prodomain comprising the N-terminally located 734 amino acids of pro-LPH, LPHα, in the folding events of LPHβinitial has been analyzed by the individual expression of both forms in COS-1 cells. Following synthesis at 37 °C LPHβinitial acquires a misfolded and enzymatically inactive conformation that is degraded by trypsin. A temperature shift to 20 °C generates a stable, trypsin-resistant, and enzymatically active LPHβinitial indicating that the individual expression of LPHβinitial results in a temperature-sensitive conformation. This form interacts at non-permissive temperatures sequentially with the ER chaperones immunoglobulin-binding protein and calnexin resulting in an ER retention. The LPHα prodomain resides in the ER when individually expressed. It reveals compact structural features that are stabilized by disulfide bridges. LPHα and LPHβinitial readily interact with each other upon coexpression, and this interaction appears to trigger the formation of a trypsin-resistant, correctly folded, enzymatically active, and transport-competent LPHβinitial polypeptide. These data clearly demonstrate that the proregion of pro-LPH is an intramolecular chaperone that is critically essential in facilitating the folding of the intermediate form LPHβinitial in the context of the pro-LPH polypeptide.

The endoplasmic reticulum (ER) is the fabric in which newly synthesized membrane-bound and secretory proteins are extensively processed and shaped to attain a competent mobility within the cell and to achieve a functional maturation. A considerable number of pivotal modification reactions commence already during the translocation of the extending polypeptide across the ER and facilitate ultimately the folding of the protein into a native conformation (for review see Ref. 1). Examples are signal sequence cleavage from the nascent polypeptide chain (2), cotranslational core N-glycosylation at particular Asn residues belonging to the sequon (Asn-X-Ser-Thr) (3), disulfide bond formation (4), and transient interactions of the folding polypeptide with a set of ER resident accessory proteins, such as molecular chaperones (5, 6). In the lumen of the ER, proteins assume their secondary and tertiary structures and, in the case of multisubunit polypeptides, assemble their individual subunits into heterodimeric, homodimeric, or larger order complexes (7). Essentially only correctly folded and assembled proteins can egress the ER and continue their further journey along the secretory pathway. The conformational status of newly synthesized polypeptides is monitored in the lumen of the ER by an efficient quality control mechanism (8). Proteins that fail to acquire a correct three-dimensional structure are retained in the ER and ultimately degraded, presumably in the cytoso1 by the ER-associated ubiquitin-proteasome pathway (9). Increasing evidence points to the possible existence of a monitoring system that operates beyond the ER. A naturally occurring mutant of intestinal sucrase-isomaltase (10) or a temperature-sensitive mutant of the G protein of vesicular stomatitis virus (11) exit the ER but are blocked in a pre-Golgi compartment instead of transported further to their final destination, the cell surface. The acquisition of a protein to a configuration similar to that of the mature and biologically active species does not always constitute an absolute requirement for its transport along the secretory pathway to the final destination. Nevertheless, retarded transport kinetics and impaired function are frequently the consequences of altered protein folding as has been demonstrated for some mutants of the hemagglutinin of the influenza virus (12) and deletion mutants of intestinal lactase-phlorizin hydrolase (13, 14). Presumably, for some proteins only correct folding of particular subdomains is adequate for their mobility along the secretory pathway. It is conceivable that the structural features of the protein critically determine whether or not a protein is retained in the ER by binding specific chaperones if it did not fulfill particular folding criteria.

Several proteins possess proregions or propeptides at the N-terminal end, which undergo cleavage during or after maturation of the precursor polypeptide. Cleavage of these propeptides is associated in many cases with biological activation of the final mature form of the protein (15) as in the cases of zymogens, neuropeptides, and prohormones (16). An important function of propeptides is that of an intramolecular chaperone that regulates and affects the folding of the precursor protein. A well-studied example is subtilisin. The propeptide of this protein comprises 77 amino acids and is critically important for...
the folding of the remaining 275 amino acids that constitute the mature and active protein (17). Human small intestinal lactase-phlorizin hydrolase (LPH, EC 3.2.1.23–26), an important component of the brush-border membrane, comprises a relatively large proregion (LPHα) that accounts for about 45% of the precursor molecule (prepro-LPH, 1927 amino acids) (18) and a brush-border mature polypeptide, LPHβ. Initial studies have demonstrated that LPH is synthesized as a single-chain polypeptide precursor, prepro-LPH, that undergoes two sequential intracellular cleavage steps: the first in the ER to pro-LPH (215-kDa) and the second, following terminal glycosylation in the Golgi apparatus, to a 160-kDa LPH (19–21).

Based on sequence analyses of various biosynthetic forms of LPH, recent studies have provided an unequivocal evidence for the existence of an additional cleavage step occurring extracellularly in the intestinal lumen (22, 23). Thus, the initial cleavage step of complex-glycosylated pro-LPH takes place at residues Arg734/Leu735, implicates a trypsin-like protease, and generates an LPH polypeptide encompassing residues 735–1927 (23) that was denoted LPHβ_initial (24). The LPHβ_initial molecule is subsequently targeted to the brush-border membrane where a final digestion by luminal trypsin occurs at Arg668/Ala669, which eliminates a peptide stretch from residues Leu735 to Arg668 yielding LPHβ_final (22, 23). This form is the polypeptide that exerts its enzymatic function as a β-galactosidase in the small intestine. The cleavage of pro-LPH to LPHβ_initial and its potential implications on the function and trafficking on this form has been studied in heterologous transfection systems. Meanwhile it has become clear that uncleaved pro-LPH undergoes a sorting event at the endoplasmic reticulum (ER) (27) by a three-way ligation. The resulting construct denoted pJB20-LPH_initial was expressed individually in the absence of sorting signals despite striking sequence homologies shared with LPHβ_initial (18). Moreover, the LPHα profragment is devoid of catalytic activity, because the lactase and phlorizin hydrolase activities have been assigned to glutamates 1271 and 3572 of LPH, respectively (28), and, moreover, the activities of the precursor pro-LPH and LPHβ_final are identical (25) for lactase or phlorizin hydrolase.

Based on the initial concepts that pro-LPH is immediately cleaved at the Arg668/Ala669 site a cDNA clone encoding the polypeptide Ala669–Phe1927, LPHβ_final, was expressed individually in the absence of the profragment, and its biosynthetic and structural features were analyzed. It was shown that the polypeptide generated was an enzymatically inactive protein, and most of it was retained as a mannosylated glycoprotein indicative of a predominant ER localization. This has lead to the hypothesis that the profragment LPHα functions as an intramolecular chaperone that is implicated in the folding of the LPHβ domain in the ER (29, 30). The intestinal form of LPHα is neither N- nor O-glycosylated, despite the presence of five potential N-glycosylation sites, and is rich in cysteine and hydrophobic amino acid residues. These features have suggested that LPHα folds rapidly into a tight and rigid globular domain in which carbohydrate attachment sites are no longer accessible to glycosyltransferases. In this paper we investigate the folding features of LPHβ and LPHβ_initial and the putative role of LPHα as an intramolecular chaperone. Additionally, in view of the new constellation that the initial cleavage occurs at Arg734/Leu735 rather than at Arg668, Ala669, it became important to determine the role of the polypeptide stretch Leu735–Arg668 in the processing and folding within the pro-LPH species.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents—**Tissue culture dishes were obtained from Greiner, Hamburg, Germany. Streptomycin, penicillin, glutamine, Dulbecco’s modified Eagle’s medium (DMEM), methionine-free DMEM (denoted Met-free medium), and trypsin were purchased from Invitrogen, Eggenstein, Germany. Fetal calf serum, pepstatin, leupeptin, aprotinin, trypsin inhibitor, and molecular mass standards for SDS-PAGE were purchased from Sigma Chemical Co., Deisenhofen, Germany. Dimethylsulfoxyl fluoride, antipain, and soybean trypsin inhibitor were obtained from Roche Diagnostics (Mannheim). Tris-HCl (1000 Ci/mmol) and protein A-Sepharose were obtained from Amersham Biosciences, Inc., Freiburg, Germany. Acrylamide, N,N′-methylenebisacrylamide, and TEMED were purchased from Carl Roth GmbH, Karlsruhe, Germany. SDS, ammonium persulfate, dithiothreitol, and Triton X-100 were obtained from Merck, Darmstadt, Germany. Endo-β-actylosulcosaminidase H (endo H) and endo-β-N-acetylglucosaminidase F/N-glycosidase F (endo FGF) were purchased from New England BioLabs, Frankfurt, Germany. The pEYFP-N1 and pECFP-N1 vectors were purchased from CLONTECH Laboratories, Inc, Heidelberg, Germany. Restriction enzymes and T4-DNA-Ligase were obtained from MBI Fermentas, St. Leon-Rot, Germany.

**Immunological Reagents—**For immunoprecipitation of human LPH mouse monoclonal antibodies (mAb) of hybridoma HBB 1/909 (20) and MLac 2, MLac 6, and MLac 8 (31) were used. The antibodies were generous gifts of Dr. Hans-Peter Hauri (Biozentrum, Basel, Switzerland), Dr. Erwin Sterchi (University of Bern, Switzerland), and Dr. Dallas Swallow (Medical Research Council, London, UK). LPHα was precipitated with the polyclonal antibody V490 directed against the N-terminal part of the profragment (30) (30). Blinost antibodies were generous gifts from Dr. Neil Bulleid, University of Manchester, UK.

**Construction of cDNA Clones—**LPHβ_initial was cloned in two steps. First, the cDNA encoding the signal sequence of pro-LPH, LPHsignal, was amplified by PCR as described before (30). A second part of the cDNA comprising the last 20 nucleotides of the LPH signal sequence (nucleotides 22–21) and nucleotides 2203–2232 of LPHα was synthesized by PCR using LPHαDNA as template and the oligonucleotides 5′-TAT CCC T-3′ (cLPH1) and 5′-GTT TTT CAT G-3′ (cLPH3600). Both DNA fragments were then fused by assembly PCR and cloned with the 3′-EcoRI/HindIII fragment of LPH cDNA into the unique EcoRI site of the pB20 expression vector (27) by a three-way ligation. The resulting construct denoted pB20-LPH_initial was sequenced and found to contain the signal sequence of pro-LPH fused in-frame to the cDNA beginning with nucleotide 2203. For the generation of a CFP fusion protein the EcoRI/SmaI fragment of pB20-LPH_initial was subcloned into the EcoRI/Smal-digested pECFP-N1 vector (CLONTECH, Inc., Heidelberg, Germany) to generate pLPH_initial-CFP.

The profragment of pro-LPH, LPHα, extending to nucleotide 2202 was first amplified from the LPHαDNA template by PCR with the primer pair LPH1 (25′-cLPH1) and LPH1 (5′-cLPH1) (AAT CTA GAG CCT CTT TAT CCC CCA G), which inserts a stop codon at position 2203 in the LPHα template. This DNA fragment was inserted into the unique EcoRI/XbaI fragment of pECFP-N1 vector (CLONTECH, Inc., Heidelberg, Germany) to generate pLPHα-CFP.

**Transient Transfection of COS-1 Cells—**Cos-1 cells were transiently transfected by DNA with using DEAE-dextran essentially as described previously (25). pB20-LPH_initial, pCDNA3-LPHα, or a mixture of both plasmids, pLPHα-CFP and pLPH_initial-CFP, were used. 48 h after transfection, the cells were biosynthetically labeled. Here, the cells were preincubated with 5 μl of methionine-free MEM for 1 h after which the medium was changed and replaced by a similar medium containing 50 μg/ml of methionine. Labeling was performed for 1 h followed by a chase with non-labeled methionine for 4 h. After labeling, the cells were rinsed twice with cold phosphate-buffered saline and solubilized with 1 ml of lysis buffer containing 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.5% sodium deoxycholate, and 0.5% TX-100 supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml peptatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml antipain, and 50 μg/ml trypsin inhibitor for 30 min at 4 °C. The cell extracts were centrifuged to remove cell debris while...
the supernatant was retained for the subsequent immunoprecipitation. After 1 h of pre-clearing with 20 μl of protein A-Sepharose, the immunoprecipitation was performed with polyclonal anti-LPHα (V496) or a mixture of mAb anti-LPH (MLac 2, MLac 6, and MLac 8) and 50 μl of protein A-Sepharose as described previously (13). For assessment of the interaction of the various LPH initial forms with BiP or calnexin, the cellular extracts were immunoprecipitated with mAb anti-BiP or mAb anti-calnexin (32). The immunoprecipitates were eluted by boiling in 1% SDS for 10 min followed by 10-fold dilution of SDS using a buffer containing 1% Triton X-100. Thereafter the samples were immunoprecipitated with a mixture of anti-LPH antibodies directed against native and denatured forms of the protein. The immunoprecipitates were further processed on SDS-PAGE.

Trypsin Treatment of Cell Lysates—LPH initial was immunoprecipitated from transient transfected COS-1 cells labeled with [35S]methionine for 6 h. The immunoprecipitates were treated with 50 μg/ml trypsin for the indicated times at 37 °C. The reaction was stopped by the addition of 2 mg/ml soybean trypsin inhibitor (Roche Molecular Biochemicals, Mannheim, Germany). Cell-free Transcription/Translation—The vector pcDNA3-LPHα containing the pro-domain of human pro-LPHα was linearized with ApaI. Transcription was initiated with T7-RNA polymerase and carried out essentially as described before (32). Subsequently, the mixture was extracted once with phenol/chloroform and twice with chloroform. After extraction once with phenol/chloroform and twice with chloroform. After extraction, the RNA was resuspended in 50 μl of RNase-free water. Translation of the newly synthesized RNA was performed in nuclease-treated rabbit reticulocyte lysate. The translation mixture comprised 10 μl of reticulocyte lysate, 1 μl of 10 mM amino acids (minus methionine), 15 μl of 100 mM 35S-methionine, and 3 μl of transcribed RNA. Where indicated, the samples were supplemented with 1 μl of nuclease-treated microsomal membranes. Translations were performed in the absence or presence of 2 or 6 mM oxidized glutathione (GSSG) at 30 °C for 60 min. Microsomal membranes were isolated as described by Bulleid and Freedman (33). Samples were prepared for electrophoresis by mixing with 5 vol. of SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, SDS (2% w/v), glycerol (10% w/v), and bromphenol blue) in the presence of 50 mM dithiothreitol for reducing conditions and for non-reducing conditions in the presence of 100 mM iodoacetamide and boiled for 5 min. After electrophoresis, gels were processed for autoradiography and exposed to Kodak X-Omat AR film.

Confocal Fluorescence Microscopy—Confocal images of living cells were acquired 2 days after transfection on a Leica TCS SP2 microscope with a ×63 water planachromat lens (Leica Microsystems) (34). Dual color CFP and YFP images were obtained by sequential scans with the 458- and 514-nm excitation lines of an argon laser and the optimal emission wavelength for CFP or YFP, respectively.

Enzymatic Assays—Lactase activity was measured according to Dahlqvist (35) using lactose as substrate. LPH α initial was immunoprecipitated from cell lysates of transiently transfected COS-1 cells. For each determination of the lactase activity, the lysate equivalent to fourteen 100-mm-diameter dishes of confluent cells was utilized. The immunoprecipitates were subsequently assayed for lactase activity essentially as described (25).

Other Procedures—Digestion of 35S-labeled immunoprecipitates with endo-β-N-acetylglucosaminidase H (endo H) and endo-β-N-acetylglucosaminidase F/glycopeptidase F (endo F/GF) was performed as previously described (21). Neuraminidase treatment of immunoprecipitates was performed in 20 mM sodium citrate, 20 mM Tris-malate buffer (pH 6.0) and protease inhibitors for 2 h at 37 °C essentially as described by Jacob et al. (27).

RESULTS

The LPH initial Polypeptide Is a Temperature-sensing Folding Mutant—The cDNA encoding the initial cleavage product, LPH initial (Leu735-Phe1927) was fused to the signal sequence of pro-LPH to allow translocation across the ER membrane (Fig. 1). Expression of this hybrid form in COS-1 cells and assessment of its glycosylation pattern using endo H sensitivity revealed a strongly labeled 150-kDa mannos-rich hydride polypeptide (LPH initial(150)) in addition to a 175-kDa endo H-resistant complex protein (LPH initial(175)). The proportions of these two forms at steady state demonstrated a predominant presence of the mannos-rich protein with ~70% of the total LPH initial protein. When LPH initial was expressed individually the amount of complex-glycosylated LPH initial was substantially reduced (30) suggesting a role of the Leu735-Arg1072 stretch in the processing of LPH initial. Nevertheless, the processing of mannos-rich LPH initial (LPH initial(c)) to the complex-glycosylated form (LPH initial(c)) was markedly less efficient in comparison with that of wild type pro-LPH (Fig. 2A) pointing to an altered folding pattern of LPH initial in the absence of the profragment. The enzymatic activity of lactase has been assigned to residues Glu1272 and Glu1429 within the LPH initial domain (36). Individual expression of this domain is associated with a drastic reduction in the lactase activity (Table I) reminiscent of a misfolded structure of LPH initial at least at or in the vicinity of the catalytic site. However, the partial conversion of LPH initial from a mannos-rich polypeptide into a complex-glycosylated protein suggests that a proportion of the LPH initial protein has acquired a correct conformation albeit at a slower rate. Alternatively, minimal folding requirements of LPH initial were fulfilled permitting the partially folded protein to egress the ER. We wanted therefore to determine whether slowing down the processing rate of LPH initial and prolonging its residence time in the ER influences the structural features of LPH initial and its biological activity. For this purpose, cells transfected with the cDNA encoding LPH initial were labeled biosynthetically at 20 °C. Fig. 2B shows that the mannos-rich polypeptide was converted to a complex-glycosylated protein after 8 h of chase. Interestingly, this protein was now as enzymatically active as its wild type pro-LPH counterpart (Ref. 25 and Table I). We
Folding of Pro-LPH and Intramolecular Chaperones

next compared the fate of LPH \( \beta_{\text{initial}} \) under the two different labeling temperatures by employing a pulse-chase protocol. Here, a dramatic difference in the turnover rates was observed. Although LPH \( \beta_{\text{initial}} \) was gradually degraded with increasing chase periods at 37°C, longer chase periods of up to 24 h at 20°C have resulted in an efficient conversion of mannose-rich LPH \( \beta_{\text{initial}}/h \) to a meanwhile predominantly labeled complex-glycosylated LPH \( \beta_{\text{initial}}/c \). This increase in stability of LPH \( \beta_{\text{initial}} \) after synthesis at 20°C was also observed by determination of the steady-state level of the polypeptide after synthesis at 37°C or 20°C in a Coomassie Blue-stained polyacrylamide gel (Fig. 2C). In these experiments LPH \( \beta_{\text{initial}}/h \) was isolated from transfected COS cells incubated at 37°C, whereas LPH \( \beta_{\text{initial}}/c \) was detected in cells cultured at 20°C. This indicates that reduced temperatures facilitate the formation of a stable conformation of complex-glycosylated LPH \( \beta_{\text{initial}}/c \). A direct comparison of the forms generated at the two different temperatures reveals an increase of ~10 kDa in the apparent molecular mass of LPH \( \beta_{\text{initial}}/c \) formed at 37°C as compared with the isoform at 20°C (Fig. 3A). One possible explanation for this difference could be altered sugar contents in both forms. To determine this content, pulse-chase experiments, with transfected cells at 37°C or 20°C combined with deglycosylation of the LPH \( \beta_{\text{initial}} \) isoforms with neuraminidase and endo F/GF, were performed (Fig. 3A). At 4 h of chase the mannose-rich glycosylated LPH \( \beta_{\text{initial}}/h \) was immunoprecipitated from the cell lysates, which was sensitive to endo F/GF and expectedly devoid of sialic acid as assessed by its insensitivity to neuraminidase treatment. Within 12 h of chase complex-glycosylated LPH \( \beta_{\text{initial}}/c \) appeared and shifted to 155 kDa after desialylation with neuraminidase. Neuraminidase also reduces LPH \( \beta_{\text{initial}}/c \) synthesized at 37°C to the 155-kDa polypeptide. Therefore, the difference in the apparent molecular masses of the LPH \( \beta_{\text{initial}} \) at both temperatures is due to a variable content of terminal sialic acid residues. To analyze if this difference in glycosylation of LPH \( \beta_{\text{initial}} \) has any influence on or is related to changes in the folding and stability of the protein, we probed its sensitivity toward trypsin. Correctly folded wild type brush-border LPH is resistant to trypsin treatment (25). Changes in the protein folding of LPH \( \beta_{\text{initial}} \) would be therefore monitored by variation in its susceptibility to trypsin. As shown in Fig. 3B, trypsin resulted in a drastic degradation of LPH \( \beta_{\text{initial}}/h \) and LPH \( \beta_{\text{initial}}/c \), which were synthesized at 37°C.

**Fig. 2.** Biosynthesis and transport kinetics of LPH \( \beta_{\text{initial}} \) in COS-1 cells. A, COS-1 cells were transiently transfected with pJB20-LPH \( \beta_{\text{initial}} \), biosynthetically labeled for 6 h followed by immunoprecipitation of LPH \( \beta_{\text{initial}} \). The immunoprecipitates were divided into three aliquots and treated with endo H, endo F/GF, or not treated. The proteins were subjected to SDS-PAGE and autoradiography. B, transiently transfected COS-1 cells were pulsed with \( \text{[}^{35}\text{S}\text{]} \)methionine for 2 h at 37°C followed by different chase times at 20°C or 37°C. Cells were lysed, and the immunoprecipitates were analyzed by SDS-PAGE and autoradiography. C, immunoprecipitates of transiently transfected COS-1 cells incubated at 20°C or 37°C were loaded onto SDS-PAGE followed by Coomassie Blue staining of the gel.

**Fig. 3.** Processing and folding of LPH \( \beta_{\text{initial}} \) at 20°C. A, COS-1 cells were transiently transfected with pJB20-LPH \( \beta_{\text{initial}} \), biosynthetically labeled at 20°C for 4 h, and chased for the indicated times or labeled at 37°C with \( \text{[}^{35}\text{S}\text{]} \)methionine for 2 h followed by a 12-h chase. After cell lysis LPH \( \beta_{\text{initial}} \) was immunoprecipitated and the immunoprecipitates were divided into equal aliquots and treated with endo F/GF, neuraminidase, or not treated. The proteins were subjected to SDS-PAGE and autoradiography. B, trypsin sensitivity assay of LPH \( \beta_{\text{initial}} \). Transiently transfected COS-1 cells were biosynthetically labeled at 37°C for 8 h or at 20°C for 12 h followed by immunoprecipitation of LPH \( \beta_{\text{initial}} \) from the cell lysates. The immunoprecipitates were treated with trypsin for different times and analyzed on SDS-PAGE. The trypsin-sensitive form of LPH \( \beta_{\text{initial}} \) detected after synthesis at 37°C is indicated by asterisks.

**TABLE I**

| Temperature | LPH \( \beta_{\text{initial}} \) | LPH \( \beta_{\text{initial}} + \text{LPH} \) |
|-------------|-------------------------------|--------------------------------------------|
| 20°C        | 2.6 ± 0.4                      | ND*                                       |
| 37°C        | 0.2 ± 0.1                      | 1.2 ± 0.4                                 |

* ND, not determined.
After 5 min of incubation with trypsin, almost all of the LPH\textsubscript{initial} was degraded. By contrast, LPH\textsubscript{initial} synthesized and processed at 20 °C was resistant to trypsin. It is worthwhile to note that a minor proportion of LPH\textsubscript{initial} synthesized at 37 °C was resistant to trypsin, and, interestingly enough, this proportion had a similar apparent molecular mass as the correctly folded LPH\textsubscript{initial} synthesized at 20 °C. The acquisition of trypsin resistance at 20 °C similar to the wild type enzyme strongly suggests that LPH\textsubscript{initial} exhibits characteristics of a temperature-sensitive protein in the absence of the LPH profragment. The variation in the folding pattern observed at 37 °C is also associated with an altered terminal glycosylation pattern.

**LPH\textsubscript{A} Alone Is a Compact Globular Polypeptide**—Obviously the presence of the LPH\textsubscript{A} profragment is crucial in influencing the folding of LPH\textsubscript{initial} in the context of wild type pro-LPH. It has been previously postulated that the LPH\textsubscript{A} molecule, which contains 11 cysteine residues, attains a compact structure that is stabilized by disulfide bridges and that acts as a kernel for the folding of the mature enzyme (30). To characterize the role of this domain in this context, we assessed its structural features in an *in vitro* translation assay. The cDNA encoding LPH\textsubscript{A} was transcribed *in vitro* and translated with rabbit reticulocyte lysate supplemented with dog pancreatic microsomal vesicles. This system has already been used to demonstrate the presence of disulfide bridges in the full-length pro-LPH enzyme (32). The main polypeptide synthesized in the absence of added microsomal vesicles had an apparent molecular mass of 90 kDa (Fig. 4A) consistent with the molecular weight of LPH\textsubscript{A} calculated from the deduced amino acid sequence (18). In the presence of microsomal membranes an additional polypeptide of 100 kDa was generated. Separation of the microsomal membranes from the reticulocyte lysates by centrifugation through a sucrose cushion revealed a predominant labeling of this species in the microsomal membranes. The 100-kDa protein is mannosie-rich and glycosylated, because it was sensitive to treatment with endo H and converted to the nascent unglycosylated 90-kDa polypeptide. The nascent 90-kDa translation product was therefore exposed to the glycosylation machinery in the lumen of the microsomal membranes. To investigate the presence of cotranslationally formed disulfide bonds, the translation reaction was carried out in the presence of oxidized glutathione (GSSG). GSSG oxidizes existing thiol groups and facilitates, therefore, the formation of disulfide bonds. It is required in the reaction mixture to compensate for the presence of the reducing agent dithiothreitol in the commercially prepared reticulocyte lysates. 2 or 6 mM GSSG was added to the reticulocyte lysates before initiation of translation to obtain a lysate that was competent in forming disulfide bonds in the newly synthesized protein. A change in the thiol/disulfide redox status of the translation products has been shown to influence the migration pattern of many proteins (37). In the absence of GSSG and under non-reducing conditions, both the 90-kDa as well as the glycosylated 100-kDa polypeptides could be detected (Fig. 4B). On the other hand, the addition of GSSG to the translation mixture results in a substantial increase in the mobility of the microsomal form of LPH\textsubscript{A} from a 100-kDa species to one of ~70 kDa under non-reducing conditions. The faster migration behavior of LPH\textsubscript{A} through the gel matrix is reminiscent of a compact conformation of this form facilitated by the formation of intramolecular disulfide bonds in the presence of GSSG. The primary sequence of LPH\textsubscript{A} contains 11 cysteine residues as putative candidates for the formation of disulfide bridges, and the obvious mobility shift observed implies that more than one disulfide bond might be formed to reach that high degree of condensation.

**LPH\textsubscript{A} Resides in the ER of Transfected COS-1 Cells**—We further analyzed the structural features of LPH\textsubscript{A} *in vivo* after expression in COS-1 cells. LPH\textsubscript{A} was immunoprecipitated from biosynthetically labeled transfected cells with the polyclonal antibody V496, which binds to the N-terminal part of LPH\textsubscript{A} (30), followed by treatment of the immunoprecipitates with endo H or endo F/GF. Fig. 5A shows that three forms of LPH\textsubscript{A} were revealed. A major 100-kDa component similar to the N-glycosylated polypeptide was synthesized in the cell-free system and two minor 103- and 97-kDa polypeptides. All these forms were converted to the non-glycosylated 90-kDa species upon N-deglycosylation with either endo H or endo F/GF, indicating that they correspond to various glycosylated isoforms of the same polypeptide. Furthermore, by virtue of the specificity of endo H in cleaving mannose-rich N-glycans and endo F/GF in cleaving mannose-rich as well as complex N-linked glycans, the data indicate that LPH\textsubscript{A} is exclusively mannosie-rich and glycosylated and is consequently located in the ER and is not further transported to the Golgi apparatus.

These findings were corroborated by confocal microscopic analyses of COS-1 cells expressing the yellow fluorescence protein (YFP) fused to LPH\textsubscript{A} (LPH\textsubscript{A}-YFP). The transfected cells revealed exclusive labeling of the ER (Fig. 5B).
activity of LPH\textsubscript{initial} expressed alone in cells cultured at 37 °C was very low, whereas this protein was enzymatically active only at the permissive temperature of 20 °C (Table 1). On the other hand, the cotransfected cells reveal a dramatic elevation in the enzymatic activity of LPH\textsubscript{initial}.

In the second approach the conformation of LPH\textsubscript{initial} in the presence or absence of LPH\textsubscript{α} was probed with trypsin to determine whether alterations in the folding pattern have occurred. LPH\textsubscript{initial} was expressed in COS cells alone or in the presence of LPH\textsubscript{α}. The biosynthetically labeled proteins were immunoprecipitated with mAb anti-LPH and treated with trypsin or with V496, the polyclonal anti-LPH\textsubscript{α} antibody. Fig. 6 shows that in the absence of cotransfected LPH\textsubscript{α} almost half of the LPH\textsubscript{initial} was cleaved by trypsin to a smaller polypeptide pair corresponding to the mannose-rich and complex-glycosylated forms. Immunoprecipitation of the cellular lysates with V496, the anti-LPH\textsubscript{α} antibody, was utilized as an internal control to confirm the absence of this protein in this experimental set up. On the other hand, coexpression of LPH\textsubscript{initial} with LPH\textsubscript{α} leads to a substantial decrease in the proportion of LPH\textsubscript{initial} cleaved by trypsin to less than 10% reminiscent of an increased stability of this protein. The presence of the LPH\textsubscript{α} protein was confirmed by its reactivity with the V496 antibody. It is established that the 160-kDa wild type brush-border LPH\textsubscript{β} is trypsin-resistant (25). That a complete resistance of LPH\textsubscript{initial} to trypsin has not been achieved is most likely due to the heterogeneous transfected cell populations, which contained both genes, or the individual ones, and therefore an optimal effect on LPH\textsubscript{initial} could be attained. This is also the reason why the enzymatic activity could not be fully restored to normal brush-border levels.

Finally, a significantly higher proportion of the complex-glycosylated species in the total synthesized LPH\textsubscript{initial} protein was detected in presence of LPH\textsubscript{α} as compared with that of the individually expressed LPH\textsubscript{initial} (69.8% \textit{versus} 79.2%). This increase suggests a more efficient processing and transport rate of mannose-rich LPH\textsubscript{initial} as a consequence of altered folding characteristics in the presence of LPH\textsubscript{α}.

In conclusion, LPH\textsubscript{initial} is a temperature-sensitive protein that is characterized by an improperly folded, trypsin-sensitive, and biologically inactive protein at 37 °C. Normal structural and functional features could be restored either at 20 °C or by addition of the prodomain LPH\textsubscript{α}, indicating that these N-terminal 734 amino acids of the proregion would assist the folding of LPH\textsubscript{initial} at 37 °C.
(LPHα-YFP). Confocal microscope imaging of transfected COS-1 cells revealed a predominant ER localization of the LPHβinitial-CFP (Fig. 7B, panel a). On the other hand, when both subunits, LPHα-YFP and LPHβinitial-CFP, were coexpressed in the same cell, LPHβinitial-CFP could be localized in the Golgi apparatus, in transport vesicles and also at the cell surface (Fig. 6B, panels b–d). Here, both species were expressed at virtually similar levels as assessed by quantification of the specific fluorescence, excluding an implicit expression of the subunits. In the absence of LPHα-YFP no significant labeling of LPHβinitial-CFP in the Golgi or at the cell surface could be observed (Fig. 7B, panel a). Together, these observations support the biochemical data, which showed that the individual expression of LPHβ results in a smaller proportion of mature and complex-glycosylated LPHβ as compared with wild type pro-LPH and LPHβ coexpressed with LPHα (see Table II, Fig. 6, and Ref. 30).

**The ER-resident Molecular Chaperones BiP and Calnexin Assist the Folding of LPHβ**—The trans effect of LPHα on the folding of LPHβinitial has led us to ask how this effect would associate and conform with other folding events that implicate ER components, such as BiP and calnexin (38). The putative and temporal interaction of LPHβinitial with BiP and calnexin was analyzed in transiently transfected COS-1 cells either expressing LPHβinitial alone or in addition of LPHα. A pulse-chase analysis of these cells followed by coprecipitation of chaperone-bound LPHβinitial with anti-BiP and anti-calnexin antibodies is demonstrated in Fig. 8. After a pulse period of 10 min the mannose-rich polypeptide of individually expressed LPHβinitial (i.e. in the absence of LPHα) could be found associated with BiP, but not calnexin. Essentially a reversed binding pattern was obtained within 30 min of chase. Here, LPHβinitial bound to calnexin and only slightly to BiP. The same pattern was also revealed after 60 min of chase. Later, at 120 min of chase, however, the earliest binding profiles of LPHβinitial to the two chaperones were repeated, i.e. strong binding to BiP and almost negligible or no binding to calnexin. Obviously LPHβinitial is subject to several cycles of association, dissociation, and reassociation with ER-resident proteins. Each of these cycles commences with the binding of BiP to an early folding intermediate of LPHβinitial followed by its dissociation and the association with calnexin, which in turn dissociates followed by the reassociation of BiP. The prolonged period of binding to these two chaperones to LPHβinitial at virtually similar intensities throughout is presumably due to an inefficient folding of LPHβinitial.

**The sequential pattern of association of LPHβinitial with BiP and calnexin changes substantially when LPHβinitial was coexpressed with LPHα.** Here, only BiP, but not calnexin, bound to LPHβinitial. The labeling intensity of LPHβinitial that was associated with BiP decreased concomitantly with the appearance of complex-glycosylated forms (Fig. 8). In sum, the data demonstrate that the absence of the LPHα prodomain exposes binding sites for calnexin resulting in the retention of partially folded forms of LPHβinitial by this chaperone and a repeated association with BiP. LPHα, on the other hand, assists the folding of LPHβinitial to a transport-competent form that leaves the ER for further transport to the cell surface. At least in part, calnexin compensates for the absence of LPHα by retaining LPHβinitial until BiP binds and the protein goes into a new folding cycle.

### Discussion

The initial intracellular proteolytic cleavage of intestinal pro-LPH in the trans-Golgi network at Arg737/Leu738 (22) is a major processing event along the secretory pathway of this protein. The generated LPHβinitial is sorted to the apical membrane, where it undergoes another cleavage step by trypsin to generate the brush-border LPH protein, LPHβfinal (22, 23). Two independent studies have previously provided strong evidence that individual expression of sequences encoding brush-border LPH, i.e. Arg367-Phe407, result in a transport-incompetent and enzymatically inactive protein (29, 30) thus implicating the profragment in the folding events of pro-LPH as an intramolecular chaperone.

In the present report we addressed the putative role of LPHα as an intramolecular chaperone as well as the interplay between this domain, the LPHβinitial, and two ER-resident proteins. These data demonstrate that LPHβinitial is a temperature-sensitive protein that folds properly to an active protein at 20 °C but is misfolded and inactive under the physiological temperature. The first folding steps of proteins destined for transport along the secretory pathway occur in the ER, which harbors a folding machinery comprising a battery of molecular chaperones. Two members of this machinery, BiP and calnexin, interact with LPHβinitial at the non-permissive temperature, whereby this interaction follows a sequential pattern and most likely proceeds for several cycles. The cycles of association, dissociation, and reassociation with the chaperone and the longer residence of the LPHβinitial protein in the ER are apparently not sufficient for the attainment of the protein to a mature, transport-competent configuration. In fact, only a minor proportion of this protein egresses the ER and acquires a complex type of glycosylation in the Golgi. Despite traversing the quality control machinery in the ER, these polypeptides are enzymatically inactive and sensitive toward trypsin treatment. This is reminiscent of altered folding that is also reflected by a variation in the N-glycosylation pattern, particularly that of the sialic acid content, as compared with the correctly folded protein. A dual function of molecular chaperones in regulating the folding of proteins has been described for many proteins. In contrast to LPHβinitial, however, the proteins in

### Table II

| Processing of LPHβinitial to a complex glycosylated protein increases in the presence of LPHα |
|-----------------------------------------------|
| COS-1 cells were transiently transfected with pJB20-LPHβinitial alone or in combination with pcDNA3-LPHα and incubated at 37 °C for 48 h. After pulse labeling for 1 h with [35S]methionine and a chase of 4 h, LPHβinitial was immunoprecipitated and analyzed by SDS-PAGE on 6% slab gels and fluorography. Results of densitometric scanning are the mean ± S.E. of five experiments. |

| LPHβinitial | LPHβinitial + LPHα | % |
|-------------|--------------------|---|
| Mannose-rich glycosylated | 30.2 ± 3.6 | 20.8 ± 3.2 |
| Complex glycosylated | 69.8 ± 3.6 | 79.2 ± 3.2 |

### Figure 8

**Sequential interaction of LPHβinitial with BiP and calnexin.** COS-1 cells were transfected with a combination of pcDNA3-LPHα and pJB20-LPHβinitial or pJB20-LPHβfinal. 48 h post transfection, the cells were labeled for 10 min followed by different chase intervals. The cell lysates were immunoprecipitated with mAb anti-LPH and when indicated with anti-BiP or anti-calnexin. The samples were analyzed on SDS-PAGE and autoradiography.
these cases acquire ultimately a correctly folded configuration. For example, the folding of the G protein of the vesicular stomatitis virus is critically dependent on the interaction of this protein with BiP and calnexin (39), whereas the immunoglobulin chains interact with BiP and GRP94 (40). The assembly of the multitransmembrane domains of the cystic fibrosis transmembrane conductance regulator (CFTR) protein in the cytosol is facilitated through the sequential binding with the chaperones hsp-2 and hsc-70 (41), and in the case of thyroglobulin the interaction with molecular chaperones follows a precursor-product relationship (42).

The defect in the folding pattern of LPH initial at the non-permissive temperature can be only overcome when the N-terminally located LPH is present. Here, enzymatic activity as well as correct folding is restored. One of the early examples of prodromains with a folding function as an intramolecular chaperone is the prodomain of subtilisin (15, 43). After maturation of the precursor molecule, this polypeptide is proteolytically cleaved to generate the biologically active protease. It can guide the folding of the inactive protein to an active protease in vitro when added exogenously (17). Intramolecular chaperone function has also been described for the prodromains of activin A (44), transforming growth factor β1 (44), cathepsin C (45), and type 1 matrix metalloproteinase (46). The 13.5-kDa N-terminal part of cathepsin C folds spontaneously and rapidly to a compact monomer with stable tertiary interactions (45). It is involved in the sequential formation of disulfide linkages in the native protein. This has also been observed for the 13-residue proregion of bovine pancreatic trypsin inhibitor, which provides an intramolecular chaperone function as a folding template for LPH initial to BiP and calnexin, in the presence of LPH, prevent the interaction of LPH initial with calnexin, suggesting that both polypeptides, LPH and calnexin, compete in binding to common sites on LPH initial. Nevertheless, calnexin by itself is not able to replace LPH, because it does not share homologies with LPH initial as LPH does and is therefore unable to behave as a folding template for LPH initial.

Acknowledgments—We thank Dr. Neil Bulleid, University of Manchester, United Kingdom, Dr. Hans-Peter Hauri, Biozentrum, University of Basel, Dr. Erwin Sterchi, Institute of Biochemistry and Molecular Biology, University of Bern, Switzerland, and Dr. Dallas Swallow, Medical Research Council, London, United Kingdom for generous gifts of monoclonal anti-BiP, anti-calnexin, and anti-LPH antibodies.

REFERENCES

1. Gething, M. J., and Sambrook, J. (1992) Nature 355, 33–45
2. von Heijne, G. (1984) J. Mol. Biol. 173, 243–251
3. Wohlfry, J. K., Shenbangamurthi, P., Lenarrz, W. J., and Naidier, F. (1983) J. Biol. Chem. 258, 11056–11063
4. Segal, M. S., Bye, J. M., Sambrook, J. F., and Gething, M. J. (1994) J. Cell Biol. 118, 227–244
5. Tatu, U., and Helenius, A. (1997) J. Cell Biol. 136, 555–565
6. Wilson, R., Lees, J. J., and Bulleid, N. J. (1998) J. Biol. Chem. 273, 9637–9643
7. Hurtley, S. M., and Helenius, A. (1989) Annu. Rev. Cell Biol. 5, 277–307
8. Hammond, C., and Helenius, A. (1994) J. Cell Biol. 121, 41–52
9. Elgaard, L., Molina, M., and Helenius, A. (1999) Science 286, 1882–1888
10. Ouwendijk, J., Moolenaar, C. E., Peters, W. J., Hollenberg, C. P., Ginsel, L. A., Fransen, J. A., and Naim, H. Y. (1996) J. Clin. Invest. 97, 633–641
11. Chen, S. S., and Huang, A. S. (1986) J. Virol. 59, 210–215
12. Tatu, U., Hammond, C., and Helenius, A. (1995) EMBO J. 14, 1340–1348
13. Jacob, R., Weiner, J. R., Stadje, S., and Naim, H. Y. (2000) J. Biol. Chem. 275, 10630–10637
14. Panzer, P., Preuss, U., Joherty, G., and Naim, H. Y. (1998) J. Biol. Chem. 273, 13861–13869
15. Barr, P. J. (1991) Cell 66, 1–3
16. Steiner, D. F., Docherty, K., and Carroll, R. (1984) J. Cell. Biochem. 24, 121–130
17. Zhu, X. L., Ohto, Y., Jordan, F., and Inouye, M. (1989) Nature 339, 483–484
18. Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunkeler, V., and Semenza, G. (1988) EMBO J. 7, 2705–2711
19. Skowmbger, H., Danielsen, U., Noreen, O., and Sjostrom, H. (1984) Biochem. Biophys. Acta 796, 247–251
20. Hauri, H. P., Sterchi, E. E., Biehn, D., Fransen, J. A., and Marzker, A. (1985) J. Cell Biol. 101, 838–851
21. Naim, H. Y., Sterchi, E. E., and Lenzte, M. J. (1987) Biochem. J. 241, 427–434
22. Jacob, R., Radebach, I., Wuthrich, M., Grunberg, J., Sterchi, E. E., and Naim, H. Y. (1996) Eur. J. Biochem. 236, 785–795
23. Wuthrich, M., Grunberg, J., Hahn, D., Jacob, R., Radebach, I., Naim, H. Y., and Sterchi, E. E. (1996) Arch. Biochem. Biophys. 336, 27–34
24. Yeh, J., and Yeh, M. (1991) J. Physiol. 260, G378–G384
25. Naim, H. Y., Lacey, S. W., Sambrook, J. F., and Getting, M. J. (1993) J. Biol. Chem. 266, 12313–12320
26. Grunberg, J., Lugnibuhl, U., and Sterchi, E. E. (1992) FEBS Lett. 314, 224–228
27. Jacob, R., Brewer, C., Fransen, J. A., and Naim, H. Y. (1994) J. Biol. Chem. 269, 2712–2721
28. Wacker, H., Keller, P., Falchetto, R., Legler, G., and Semenza, G. (1992) J. Biol. Chem. 267, 18744–18752
29. Oberholzer, T., Mantei, N., and Semenza, G. (1996) FEBS Lett. 333, 127–131
30. Naim, H. Y., Jacob, R., Naim, H., Sambrook, J. F., and Getting, M. J. (1994) J. Biol. Chem. 269, 26933–26943
31. Mauiri, L., Ruia, V., Potter, J., Swallow, D., Ho, M. W., Fiocca, R., Finzi, G., Cornaggi, M., Capella, C., and Quaroni, A. (1991) Gastroenterology 100, 359–369
32. Jacob, R., Bulleid, N. J., and Naim, H. Y. (1995) J. Biol. Chem. 270, 19678–19684
33. Bulleid, N. J., and Freedman, R. B. (1988) Nature 335, 649–651
34. Jacob, R., and Naim, H. Y. (2001) Curr. Biol. 11, 1444–1450
35. Dahlqvist, A. (1968) Anal. Biochem. 22, 99–107
36. Zeeva, L., Mesonero, J. E., Stutz, A., Poirier, J. C., Giudicelli, J., Cursio, R.,
Gloor, S. M., and Semenza, G. (1998) FEBS Lett. 435, 225–228
37. Goldenberg, D. P., and Creighton, T. E. (1984) Anal. Biochem. 138, 1–18
38. High, S., Lecomte, F. J., Russell, S. J., Abell, B. M., and Oliver, J. D. (2000) FEBS Lett. 476, 38–41
39. Hammond, C., and Helenius, A. (1994) Science 266, 456–458
40. Melnick, J., Dul, J. L., and Argon, Y. (1994) Nature 370, 373–375
41. Meacham, G. C., Lu, Z., King, S., Sorscher, E., Touson, A., and Cyr, D. M. (1999) EMBO J. 18, 1492–1505
42. Kim, P. S., and Arvan, P. (1995) J. Cell Biol. 128, 29–38
43. Inouye, M. (1991) Enzyme 45, 314–321
44. Gray, A. M., and Mason, A. J. (1990) Science 247, 1328–1330
45. Cigic, B., Dahl, S. W., and Pain, R. H. (2000) Biochemistry 39, 12382–12390
46. Cao, J., Hymowitz, M., Conner, C., Bahou, W. F., and Zucker, S. (2000) J. Biol. Chem. 275, 29648–29653
47. Weissman, J. S., and Kim, P. S. (1992) Cell 71, 841–851
48. Allen, S., Naim, H. Y., and Bulleid, N. J. (1995) J. Biol. Chem. 270, 4797–4804
The Prosequence of Human Lactase-Phlorizin Hydrolase Modulates the Folding of the Mature Enzyme

Ralf Jacob, Karen Peters and Hassan Y. Naim

J. Biol. Chem. 2002, 277:8217-8225.
doi: 10.1074/jbc.M111500200 originally published online December 18, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M111500200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 48 references, 22 of which can be accessed free at http://www.jbc.org/content/277/10/8217.full.html#ref-list-1