Characterization of Three Fragments That Constitute the Monomers of the Human Lysyl Hydroxylase Isoenzymes 1–3

THE 30-kDa N-TERMINAL FRAGMENT IS NOT REQUIRED FOR LYSYL HYDROXYLASE ACTIVITY*

Received for publication, December 18, 2001, and in revised form, April 10, 2002
Published, JBC Papers in Press, April 15, 2002, DOI 10.1074/jbc.M112077200

Kati Rautavuoma, Kati Takaluoma, Kaisa Passoja, Asta Pirskanen, Ari-Pekka Kvist, Kari I. Kivirikko, and Johanna Myllyharju†

From the Collagen Research Unit, Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, University of Oulu, FIN-90014 Oulu, Finland

Lysyl hydroxylase (LH, EC 1.14.11.4), a luminally oriented peripheral membrane protein within the endoplasmic reticulum, catalyzes the hydroxylation of lysine in collagens and more than 15 other proteins by the hydroxylation of -X-Lys-Gly- sequences, for which it requires Fe²⁺, 2-oxoglutarate, O₂, and ascorbate (1, 2). The resulting hydroxylysine residues have two important functions. They are essential for the stability of the intermolecular collagen cross-links, and their hydroxy groups serve as attachment sites for carbohydrate units, either the monosaccharide galactose or the disaccharide glucosylgalactose (1). The functions of the hydroxylysine-linked carbohydrate units are not fully understood, but in the case of fibril-forming collagens they influence the lateral packing of collagen molecules into fibrils and the fibril diameters (1, 3). Hydroxylysine residues appear to play an especially critical role in the type IV collagens of basement membranes, because mutations in the gene for the only LH present in the nematode Caenorhabditis elegans, which is involved in the synthesis of basement membrane collagens, are embryonic lethal (4).

Three isoenzymes of human LH have been cloned so far (5–10); the sizes of the processed human LH1, LH2, and LH3 polypeptides are 709, 712, and 714 amino acid residues, respectively (5–9). The highest degree of sequence identity was found within the catalytically important C-terminal regions, which contain the two histidines and one aspartate (11–13) that provide the three ligands for the binding of Fe²⁺ to the catalytic site and the arginine that binds the C-5 carboxyl group of the 2-oxoglutarate (14). The catalytic subunit of the other main collagen hydroxylase, prolyl 4-hydroxylase, has a separate peptide substrate-binding domain that is distinct from the catalytic domain (15), but no data are available to indicate whether LH also has a separate peptide-binding domain.

All three LH isoenzymes have been expressed as recombinant proteins in insect cells, and the recombinant enzymes present in crude insect cell extracts have been shown to hydroxylate lysine residues with similar kinetic properties (7–9, 11, 16). Very surprisingly, however, recombinant LH3 was also found to possess collagen glucosyltransferase activity but not LH1 or LH2 (17). No data are available on the level of this activity in the LH3 polypeptide nor on the possible biological significance of the finding (17). It is also unknown whether this isoenzyme also possesses collagen galactosyltransferase activity, as the glucose residue is only transferred to a galactosyl-

† To whom correspondence should be addressed: Dept. of Medical Biochemistry and Molecular Biology, P. O. Box 6000, University of Oulu, FIN-90014 Oulu, Finland. Tel:358-8-537-5740; Fax:358-8-537-5811; E-mail:johanna.myllyharju@oulu.fi.

‡ This work was supported by grants from the Health Science Council of the Academy of Finland, the Finnish Centre of Excellence Programme 2000–2005 Grant 44843, and FibroGen Inc. (South San Francisco, CA). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
to the domain structure of the enzyme, and whether the enzyme also has collagen galactosyltransferase activity.

MATERIALS AND METHODS

Construction of Baculovirus Transfer Vectors and Generation of Recombinant Baculoviruses—A full-length LH2 cDNA (7) was created here by amplifying two cDNA fragments by PCR, the first one corresponding to nucleotides 1–534 of the coding sequence, with a NotI site preceding the translation start codon, and the second one containing the coding sequence from nucleotide 512 to the translation stop codon, followed by 210 bp of 3′-untranslated sequence and an XhoI site. These fragments were obtained from human placenta and pancreas Marathon-Ready cDNAs (CLONTECH), respectively. The LH2 cDNA fragments were digested with NotI-PolI and PolI-XhoI, respectively, and ligated to NotI-XhoI-digested pBluescript. A BamHI site and one cyto- sine and six histidine codons were inserted by PCR between the codons for the last amino acid of the signal peptide and the first amino acid of the processed LH polypeptide in the full-length cDNAs for LH1 (5), LH2, and LH3 (8). The Hisx6-LH1 cDNA was digested with BamHI-EcoRI; the Hisx6-LH2 and Hisx6-LH3 cDNAs were digested with BamHI-XhoI, and the inserts were ligated to pAcGP67A (BD PharMingen). The constructs thus contain a baculovirus GP67 signal sequence, a histidine tag, and cDNAs coding for the processed LH polypeptide.

LH fragment constructs with an N-terminal histidine tag were generated by amplifying cDNA fragments corresponding to amino acids 1–262, 255–572, and 565–709 of LH1 and 1–266, 261–577, and 570–714 of LH3. Each fragment contained a BamHI site, one cytosine and six histidine codons in the 5′ end, and a translation stop codon and an EcoRI site in the 3′ end. Fragment pair constructs corresponding to amino acids 1–572 and 255–709 of LH1 and 1–577 and 261–714 of LH3 were generated in a similar way and ligated to BamHI-EcoRI-digested pAcGP67A (BD PharMingen).

The sequences were verified by DNA sequencing on an automated device (ABI Prism 377, Applied Biosystems, Inc.) using BigDye or dRhodamine Terminator Cycle Sequencing Ready Reaction kits (PE Biosystems).

The recombinant baculovirus vectors were co-transfected into Spodoptera frugiperda SF9 cells with modified Autographa californica nuclear polyhedrosis virus DNA (BD PharMingen) by calcium phosphate transfection, and the recombinant viruses were selected (18).

Expression of Recombinant Proteins in Insect Cells and Their Purification—High Five insect cells (Invitrogen) were cultured as monolayers in T75-FH medium (Sigma) supplemented with 10% fetal bovine serum (ICN) or in suspension in SD900IISFM serum-free medium (In VitroGen). The cells, seeded at a density of 5 × 10^6 cells/100-mm plate and 1 × 10^7/ml, were infected at a multiplicity of 5 with the viruses coding for the histidine-tagged full-length LH1, LH2, or LH3 or the LH1 and LH3 fragments and fragment pairs. The cells were harvested 48 h after infection, washed, and homogenized as described before (8, 11). Aliquots of the soluble fractions of the cell homogenates were analyzed by 8 or 12% SDS-PAGE under reducing conditions, and the cell pellets were further solubilized in 1% SDS and analyzed in the same manner. To purify the histidine-tagged full-length LH1, LH2, and LH3 and the LH1 and LH3 fragments and fragment pairs, the media from the suspension cultures were harvested by centrifugation at 2,500 rpm for 10 min, and samples were incubated with ProBond metal chelate affinity resin (Invitrogen) pre-equilibrated with 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol, and 0.02 M Tris buffer, pH 7.5, at 4 °C overnight with gentle mixing. The amount of ProBond used was 2 ml per 100-ml medium sample. The ProBond resin was packed into a column and washed with 10 volumes of the above buffer, and the bound proteins were eluted with the same buffer containing 0.2 or 0.4 M imidazole. Fractions of 3 ml were collected and analyzed by 12% SDS-PAGE under reducing conditions. Fractions containing the full-length LH polypeptides or the fragments or fragment pairs were pooled, and the samples were passed through a PD-10 column (Amerham Biosciences) equilibrated with 0.2 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, 1% glycerol, and 0.02 M Tris buffer, pH 7.5, to remove the imidazole.

The protein concentrations were measured with a Bio-Rad protein assay kit (Bio-Rad) according to the manufacturer’s instructions. The molecular weights of the full-length LH isoenzymes were determined by gel filtration in a calibrated HiLoad 16/60 Superdex 200 column (Amerham Biosciences), equilibrated, and eluted with 0.2 M NaCl, 0.1 M glycine, 1% glycerol, and 0.02 M Tris buffer, pH 7.5.

Protease Digestion of Full-length LH Polypeptides—Purified full-length recombinant human LH1, LH2, or LH3, 10–100 μg, was digested with thermolysin, trypsin, or proteinase K at a protease:LH ratio of 1:100 to 1:130 °C for 30–90 min, and the digestion was stopped by the addition of Pefabloc SC (Roche Molecular Biochemicals) or EDTA (for thermolysin) to 3 mM. Samples were analyzed by 8% SDS-PAGE in A and 8% non-denaturing PAGE in B, followed by staining with Coomassie Blue in both panels. Lanes 1, LH1; lanes 2, LH2; lanes 3, LH3; lane 4 (in B), prolyl 4-hydroxylase.

FIG. 1. Analysis of the purified recombinant LH isoenzymes by SDS-PAGE under reducing conditions and by non-denaturing PAGE. High Five insect cells cultured in suspension were infected with baculoviruses coding for histidine-tagged LH polypeptides. Medium samples were harvested 48 h after infection, and the polypeptides were purified as described under “Materials and Methods.” The samples were analyzed by 8% SDS-PAGE in A and 8% non-denaturing PAGE in B, followed by staining with Coomassie Blue in both panels. Lanes 1, LH1; lanes 2, LH2; lanes 3, LH3; lane 4 (in B), prolyl 4-hydroxylase.

Purification of Full-length Histidine-tagged LH Polypeptides—The use of a baculovirus signal peptide GP67 has been reported previously (16) to lead to efficient secretion of a recombinant LH1 polypeptide expressed in insect cells. In order to develop a simple method for purifying LH polypeptides, cDNAs coding for the processed LH1, LH2, and LH3 polypeptides were cloned into a pAcGP67A expression vector, and a histidine tag was inserted between the GP67 signal sequence and the LH cDNA in each case. High Five insect cells cultured in suspension were infected with baculoviruses coding for the histidine-tagged LH1, LH2, or LH3 polypeptides. Medium samples were harvested 48 h after infection and incubated with a ProBond metal chelate affinity resin, and the unbound proteins were washed off with the column equilibration buffer, and the bound proteins were eluted with imidazole. The fractions were analyzed by SDS-PAGE and were found to contain polypeptides with sizes of 80–85 kDa, corresponding to full-length LH1, LH2, or LH3. The identities of the polypeptides were verified by N-terminal sequencing (data not shown). The fractions containing the LH1, LH2, or LH3 polypeptides were pooled; imidazole was removed in a PD-10 column, and the purified samples were analyzed by SDS-PAGE (Fig. 1A).
nondenaturing PAGE (Fig. 1B). The final preparations were found to be pure after one-step metal chelate affinity purification (Fig. 1) and to have LH activity (as shown for LH1 in Table I). Gel filtration experiments in a calibrated column indicated that all three LH isoenzymes had identical molecular weights, about 180,000 (details not shown).

Limited Proteolysis of the LH Polypeptides—In order to identify possible domain structures of the human LH1, LH2, and LH3 polypeptides, the purified recombinant enzymes were subjected to limited proteolysis with thermolysin, trypsin, or proteinase K for 30–90 min at 37 °C. The samples were analyzed by 12% Tris-Tricine PAGE and Coomassie staining. Five major protease-resistant peptides of about 68, 37, 33, 18, and 16 kDa were found after digestion of the LH polypeptides with thermolysin, trypsin, and proteinase K (as shown for a thermolysin-digested sample of LH1 in Fig. 2), except that the 18-kDa fragment was not obtained for LH3 (data not shown).

N-terminal sequencing of the major protease-resistant LH1 peptides showed that the N terminus of the 68- and 33-kDa peptides contained the histidine tag, whereas the N-terminal amino acid of the 37-kDa peptide was either Gly-261, Ile-262 (Fig. 3A), or Asp-253 (protease K, data not shown) depending on the protease used, and those of the 18- and 16-kDa peptides were either Ile-571 (Fig. 3A) or Gly-573 (protease K). The N-terminal amino acids of the corresponding protease-resistant LH3 peptides were a histidine from the affinity tag, Leu-241 or Thr-264 and Leu-576 (Fig. 3A) or Asp-253 (proteinase K, data not shown) depending on the protease used, and those of the 18- and 16-kDa peptides were either Gly-576 (protease K, not shown). The data would be consistent with the presence of at least two main protease-sensitive regions in LH polypeptides and would suggest the existence of at least three structural domains, termed fragments A, B, and C, respectively (Fig. 3B). The calculated molecular masses with these cleavage sites are 29.6, 36.2, and 15.9 kDa for the nonglycosylated LH1 fragments and 30.4, 36.2, and 15.8 for the LH3 fragments. The 68-kDa polypeptide corresponds to fragment pair A-B, the 33-kDa polypeptide to fragment A with efficient utilization of its two N-glycosylation sites, and the 37-kDa polypeptide represents fragment B, whereas the 18-kDa peptide obtained only from LH1 is probably due to utilization of the N-glycosylation site present at residue 668 in the 16-kDa fragment C of LH1 but not LH3. The N-terminal amino acids of the protease-resistant LH2 peptides were not determined, but in view of their similarity in size to the protease-resistant LH1 and LH3 peptides, these sites were assumed to be located in the corresponding regions.

Protease cleavages at some additional sites were also occasionally observed in some of the purified LH samples, mainly when a 1:10 protease to LH ratio was used (Fig. 3A). No such additional sites were identified with either thermolysin or trypsin within regions B and C (Fig. 3), whereas two additional protease-sensitive sites were found within region A in some experiments, but not all, and therefore this region may in fact consist of up to three domains (Fig. 3). However, as these minor sites were seen only in some experiments, they may also represent protease-sensitive sites within a folded domain. Limited proteolysis with proteinase K identified a few additional sites in some experiments that were not sensitive to thermolysin or trypsin and are therefore not likely to represent additional domain boundaries (details not shown).

Expression of Recombinant LH Fragments in Insect Cells and Their Purification—In order to study whether it is possible to express the recombinant LH1 and LH3 fragments A–C in folded forms, recombinant pAcGp67A vectors coding for the LH1 amino acids 1–262, 255–572, and 565–709 and the LH3 amino acids 1–266, 261–577, and 570–714 were generated. Each construct contained sequences coding for a histidine tag between those coding for the GP67 signal sequence and the LH fragments. Recombinant viruses coding for the LH fragments were used to infect High Five insect cells in suspension, and the cells and medium samples were harvested 48 h after infection. The cells were homogenized in a buffer containing 1% Nonidet P-40 and centrifuged, and the cell pellet was homogenized further in a buffer containing 50% glycerol (11), incubated on ice, and centrifuged. The remaining pellet was solubilized in 1% SDS, and the samples were analyzed by SDS-PAGE. All recombinant LH fragments were found to be partly soluble in the glycerol-containing buffer, but the highest amounts were found in the SDS-soluble fraction (details not shown). Similar data have been reported previously (8, 11) for the full-length LH1 and LH3 polypeptides. Fragments A of LH1 and LH3 were secreted into the culture medium and could be purified to homogeneity from medium samples as described above for the full-length polypeptides (Fig. 4A, lanes 1 and 2). In contrast, the levels of fragments B and C in the culture medium were too low for attempts to purify them to homogeneity (details not shown).

To study whether it is possible to express the LH1 and LH3 fragment pairs A-B and B-C in folded forms, recombinant pAcGp67A vectors coding for the LH1 amino acids 1–572 and 255–709 and the LH3 amino acids 1–577 and 261–714 were generated with N-terminal histidine tags, and the recombinant viruses were used to infect High Five insect cells in suspension. A significant portion of these fragment pairs was found to be secreted into the culture medium and could be purified to homogeneity as above (Fig. 4A, lanes 2, 3, 5, and 6).

Urea Gradient Gel Electrophoresis and Circular Dichroism Analysis of Purified Recombinant LH Fragments—To study whether the purified recombinant A fragments and the A-B and B-C pairs were folded, purified samples were analyzed by urea gradient gel electrophoresis and circular dichroism spectroscopy. Urea gradient gels indicated that the recombinant LH1 and LH3 A fragments and fragment pairs were fully folded, as a sigmoidal transition in mobility resulting from unfolding along the urea gradient was observed in all cases, as shown in Fig. 4B for LH1 fragment A. The far-UV CD spectra of the purified recombinant LH1 and LH3 A fragments and the A-B and B-C pairs were also typical of a folded protein (Fig. 5).

Lysyl Hydroxylase Activity of Purified Recombinant LH1 and LH3 and Their Recombinant Fragments—The purified recombinant full-length LH1 and LH3 isoenzymes and their recombinant fragments were analyzed for lysyl hydroxylase activity with an assay based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate with the synthetic peptide (Ile-Lys-Gly)₃ as a substrate (19). As reported previously (17), the imidazole that was used to elute the nickel affinity column

| Polypeptide | Lysyl hydroxylase activity (dpm/nmol) |
|------------|--------------------------------------|
| LH1        |                                      |
| Expression set 1 | 116,000                             |
| Expression set 2 | 94,000                              |
| B–C        |                                      |
| Expression set 1 | 83,000                              |
| Expression set 2 | 142,000                             |
| A          | 0                                    |
| A–B        | 0                                    |
rapidly abolished the LH activity of the full-length enzymes and their fragment pairs, especially those of LH3. Consequently, activity values are given in Table I only for LH1. As expected, the recombinant LH1 and LH3 A fragments and A-B pairs that lacked the C-terminal catalytic region had no LH activity (as shown for LH1 in Table I). Surprisingly, the recombinant LH1 pair B-C, which contains all the critical residues required for cosubstrate binding and lacks the N-terminal 30-

**FIG. 2.** Tris-Tricine PAGE analysis of LH1 peptides resisting limited proteolysis with thermolysin. Lane 1, undigested LH1; lane 2, LH1 digested with thermolysin at 37 °C for 60 min. The amino acid sequences on the right were obtained using N-terminal sequencing.

**FIG. 3.** Protease-sensitive regions in the LH isoenzymes. A, thermolysin and trypsin-sensitive sites within the amino acid sequences of the processed human LH1, LH2, and LH3 polypeptides. White letters on a black background indicate identity, and black letters on a gray background indicate similarity. The arrows indicate the N-terminal amino acids of the protease-resistant LH1 or LH3 peptides after digestion with thermolysin (open arrows) or trypsin (filled arrows). Positions of the two main protease-sensitive regions are underlined. Residues required for the binding of the Fe(II) atom and the C-5 carboxyl group of 2-oxoglutarate are indicated by asterisks. B, schematic representation of the protease-sensitive regions that may correspond to the domain structure of the LH polypeptides. The positions of the two main protease-sensitive regions are indicated by arrows and those of two additional sites found within fragment A by dashed arrows.
A fragment, was a fully active lysyl hydroxylase (Table I). The LH3 pair B-C lost its activity particularly rapidly. The activity levels obtained with this fragment pair were nevertheless found in some purification experiments to be identical to those measured for the full-length LH3 purified at the same time (details not shown), indicating that the B-C pair of LH3 is also a fully active enzyme. The values of the LH1 B-C pair for the peptide substrate and the cosubstrates Fe$_{2}^+$/H$_{11001}$ sphere glutarate, and ascorbate were essentially identical to those of the full-length LH1 and LH3 enzymes (Table II).

Collagen Glycosyltransferase Activities of the Purified Recombinant LH1 and LH3 and Their Recombinant Fragments—

The purified recombinant full-length LH1 and LH3 isoenzymes and their recombinant fragments and fragment pairs were analyzed for collagen glycosyltransferase activity by a method based on the transfer of $^{14}$C-glucose from radioactive UDP-glucose to galactosylhydroxylysine residues in a denatured citrate-soluble rat skin collagen substrate (19, 21). As reported previously (17), full-length recombinant LH3 showed collagen glycosyltransferase activity but not LH1 (Table III). However, the activity level found was only about 2% of that previously obtained with purified collagen glycosyltransferase from chick embryos (19, 23). The recombinant N-terminal LH3 A fragment and the LH3 A-B pair also had collagen glycosyltransferase activity, whereas the LH3 B-C pair had no such activity (Table III).

To verify that the glucosyltransferase activity assay did not give markedly low values due to some artifact, we also assayed this activity level in two human serum samples. The amount of collagen glycosyltransferase activity in serum samples from healthy human subjects has been reported previously to range from 0.24 to 0.60 milliunit/ml (milliunit being defined as described previously (24)). The values obtained for the two serum samples assayed here together with the LH3 and its fragment preparations were 0.32 and 0.36 milliunit/ml, i.e. well within the range of the previously reported control values. This indicates that the glucosyltransferase activity assay gave values similar to those reported previously (23, 24).

The $K_m$ values of LH3 for UDP-glucose and the denatured citrate-soluble rat skin collagen substrate were about 150 $\mu$M and 4 g/liter, respectively (details not shown). Both values are higher than the values of 5–30 $\mu$M reported for UDP-glucose with collagen glycosyltransferases from various sources and 0.5–1 g/liter reported for denatured citrate-soluble rat skin

![Fig. 4. Analysis of purified recombinant LH fragments A and fragment pairs A-B and B-C by SDS-PAGE under reducing conditions and demonstration of the folding of the purified recombinant LH1 A fragment with transverse urea-gradient gel electrophoresis. A, High Five insect cells cultured in suspension were infected with baculoviruses coding for histidine-tagged LH fragments and fragment pairs. Medium samples were harvested 48 h after infection, and the polypeptides were purified as described under “Materials and Methods.” Lane 1, fragment A of LH1; lane 2, A-B of LH1; lane 3, B-C of LH1; lane 4, A of LH3; lane 5, A-B of LH3; lane 6, B-C of LH3. B, fragment A of LH1, 50 $\mu$g, was run with a urea gradient from 0 to 8 M perpendicular to the direction of electrophoresis, which was performed at 150 V for 4 h at 4 °C. Proteins were visualized by Coomassie Blue staining.

![Fig. 5. Far-UV CD-spectra of the purified recombinant LH1 and LH3 fragments and fragment pairs. Purified recombinant LH1 and LH3 fragments (A) and fragment pairs A-B (B) and B-C (C) were analyzed, and the CD spectra of the LH3 domains are presented with thick lines and those of LH1 with thin lines.](http://www.jbc.org/)

### Table II

| Substrate or cosubstrate | $K_m$ (LH1) | $K_m$ (LH1:B-C) | $K_m$ (LH3) |
|--------------------------|------------|----------------|------------|
| Fe$_{2}^+$               | 5          | 4              | 1          |
| 2-Oxoglutarate           | 120        | 140            | 100        |
| Ascorbate                | 350        | 330            | 350        |
| (IRG)$_{3}$              | 400        | 380            | 430        |
The present paper reports for the first time on the isolation of the three recombinant human LH isoenzymes 1–3 as homogeneous proteins. LH1 has been isolated previously as a homogeneous protein from chick embryos and human placental tissues by two affinity column procedures (30–32). Attempts to purify recombinant human LH1 expressed in the endoplasmic reticulum of insect cells were hampered by the marked insolubility and aggregation tendency of the recombinant enzyme (11), properties that have also made it difficult to isolate any large amounts of non-recombinant LH1 (30, 31). Recombinant LH3 was found previously to be more soluble than LH1 (8), but no attempts have been reported to purify either LH3 or LH2 to homogeneity. A key feature in the present purification procedure was the use of the signal sequence of the baculoviral glycoprotein GP67 (16). This led to the secretion of the three recombinant LH isoenzymes into the insect cell culture medium, in which the recombinant enzymes were not present in membrane-bound forms and showed a less marked aggregation tendency than inside the endoplasmic reticulum. Another key feature was the use of a histidine tag, which remained in the N termini of the processed enzymes and made it possible to purify the isoenzymes to homogeneity with simple steps. Unfortunately, the imidazole that is used to elute the nickel affinity column rapidly abolishes lysyl hydroxylase activity (17), especially that of the LH3 polypeptide, whereas the glucosyltransferase activity of this same polypeptide is not affected (17). It was therefore not possible to isolate the LH isoenzymes with maximal catalytic activity by this procedure. The highest catalytic center activity measured for any recombinant lysyl hydroxylase preparation here, when calculated from the amount of 2-oxo[1-14C]glutarate decarboxylated and corrected to saturating concentrations of 2-oxoglutarate and the peptide substrate, was 65 mmol/mol/min, but in most cases the values were only about 10–15 mmol/mol/min. The highest catalytic center activities measured for non-recombinant LH1 isolated by a procedure involving the use of two affinity columns have reached to 60–100 mmol/mol/min (Ref. 30, recalculated for one catalytic site/enzyme monomer), but the non-recombinant enzyme also becomes rapidly inactivated, and the catalytic center activities of most preparations have been much lower (19, 30).

LH1 isolated from chick embryos and human placental tissues has been shown by gel filtration to be a homodimer with an apparent molecular weight of about 180,000 (30–32). The present gel filtration data indicate that all three LH isoenzymes are homodimers with similar molecular weights, so that the differences in their mobilities seen in analyses by non-denaturing PAGE (Fig. 1B) are likely to represent differences in the charges of the enzymes rather than in their molecular weights. The calculated pI values for LH1, LH2, and LH3 are 6.47, 6.14, and 5.56, respectively.

Limited proteolysis experiments indicated the presence of at least two protease-sensitive regions in the LH polypeptides, suggesting that the polypeptides may consist of at least three distinct domains. However, only the N-terminal recombinant fragment A was secreted in significant amounts into the culture medium, whereas the recombinant B and C fragments mainly accumulated inside the insect cells. Interestingly, both recombinant double-fragment polypeptides A-B and B-C were secreted and could be purified to homogeneity. It was thus possible to study the properties of one recombinant fragment and two recombinant fragment pairs. All these recombinant polypeptides were folded, as judged by urea gradient gel electrophoresis and CD spectrum analysis, suggesting that they may indeed represent distinct domains.

A highly surprising finding was that the N-terminal fragment A plays no critical role in the catalytic activity of LH, because the recombinant B-C polypeptide, lacking fragment A, was a fully active hydroxylase. Furthermore, the $K_m$ values

### Table III

| Polypeptide                              | Glucosyltransferase activity mmol/mol/min |
|------------------------------------------|------------------------------------------|
| LH1                                      | <40                                      |
| LH3                                      |                                          |
| Preparation 1                            | 880                                      |
| Preparation 2                            | 920                                      |
| LH3-A                                    |                                          |
| Preparation 1                            | 510                                      |
| Preparation 2                            | 750                                      |
| LH3-B                                    |                                          |
| Preparation 1                            | 450                                      |
| Preparation 2                            | 530                                      |
| LH3-B-C                                  |                                          |
| Preparation 1                            | 450                                      |
| Preparation 2                            | 530                                      |
| Chick-embryo collagen glucosyltransferase | 45,900                                   |

*See Ref. 23.*

collagen with collagen glucosyltransferase from chick embryos (19).

LH3 was also found to have collagen galactosyltransferase activity. However, the level of this activity was very low and was in some LH3 preparations below the limit of reliable detection. The mean activity level in three preparations with the highest activity levels was 63 mmol/mol/min (range 44–98 mmol/mol/min), whereas the corresponding mean for the recombinant LH3 fragment A was 20 mmol/mol/min (range 16–25 mmol/mol/min, details not shown). These activity values may be specific, as no galactosyltransferase activity was found in any of the five LH1 preparations and one LH3 B-C pair preparation assayed for this activity (details not shown). The activity levels were so low, however, that no studies could be carried out to establish the possible specificity.

### DISCUSSION

It has been known for 3 decades that a deficiency in lysyl hydroxylase activity leads to a heritable connective tissue disorder known as the kyphoscoliotic type (25) of the Ehlers-Danlos syndrome (26). The clinical phenotypes are characterized by bruisable skin, joint laxity, kyphoscoliosis, muscle hypotonia, and ocular abnormalities such as fragility of the globe and spontaneous retinal detachment (1, 2, 25–28). For a long time it was not understood why these patients lacked hydroxylase activity leads to a heritable connective tissue disorder, as mutations in the only LH gene present in C. elegans (1, 28). It seems highly likely that mutations of the gene for LH1 (1, 28). It seems highly likely that mutations of the gene for the other LH isoenzymes may have even more serious consequences, as mutations in the only LH gene present in C. elegans, which is involved in the synthesis of type IV collagen, are embryonic lethal (4). Little is currently known about the other two LH isoenzymes, however, and nothing is known of their mutations. Evidence has even been presented suggesting the existence of a fourth human LH isozyme (29), which hydroxylates only lysine residues in telopeptides, the short non-triple helical sequences at both ends of collagen molecules, but no attempts to clone or characterize this isozyme have been reported so far.

The present paper reports for the first time on the isolation of the three recombinant human LH isoenzymes 1–3 as homogeneous proteins. LH1 has been isolated previously as a homogenous protein from chick embryos and human placental tissues by two affinity column procedures (30–32). Attempts to purify recombinant human LH1 expressed in the endoplasmic reticulum of insect cells were hampered by the marked insolubility and aggregation tendency of the recombinant enzyme (11), properties that have also made it difficult to isolate any large amounts of non-recombinant LH1 (30, 31). Recombinant LH3 was found previously to be more soluble than LH1 (8), but no attempts have been reported to purify either LH3 or LH2 to homogeneity. A key feature in the present purification procedure was the use of the signal sequence of the baculoviral glycoprotein GP67 (16). This led to the secretion of the three recombinant LH isoenzymes into the insect cell culture medium, in which the recombinant enzymes were not present in membrane-bound forms and showed a less marked aggregation tendency than inside the endoplasmic reticulum. Another key feature was the use of a histidine tag, which remained in the N termini of the processed enzymes and made it possible to purify the isoenzymes to homogeneity with simple steps. Unfortunately, the imidazole that is used to elute the nickel affinity column rapidly abolishes lysyl hydroxylase activity (17), especially that of the LH3 polypeptide, whereas the glucosyltransferase activity of this same polypeptide is not affected (17). It was therefore not possible to isolate the LH isoenzymes with maximal catalytic activity by this procedure. The highest catalytic center activity measured for any recombinant lysyl hydroxylase preparation here, when calculated from the amount of 2-oxo[1-14C]glutarate decarboxylated and corrected to saturating concentrations of 2-oxoglutarate and the peptide substrate, was 65 mmol/mol/min, but in most cases the values were only about 10–15 mmol/mol/min. The highest catalytic center activities measured for non-recombinant LH1 isolated by a procedure involving the use of two affinity columns have reached to 60–100 mmol/mol/min (Refs. 30 and 31, recalculated for one catalytic site/enzyme monomer), but the non-recombinant enzyme also becomes rapidly inactivated, and the catalytic center activities of most preparations have been much lower (19, 30).

LH1 isolated from chick embryos and human placental tissues has been shown by gel filtration to be a homodimer with an apparent molecular weight of about 180,000 (30–32). The present gel filtration data indicate that all three LH isoenzymes are homodimers with similar molecular weights, so that the differences in their mobilities seen in analyses by non-denaturing PAGE (Fig. 1B) are likely to represent differences in the charges of the enzymes rather than in their molecular weights. The calculated pI values for LH1, LH2, and LH3 are 6.47, 6.14, and 5.56, respectively.

Limited proteolysis experiments indicated the presence of at least two protease-sensitive regions in the LH polypeptides, suggesting that the polypeptides may consist of at least three distinct domains. However, only the N-terminal recombinant fragment A was secreted in significant amounts into the culture medium, whereas the recombinant B and C fragments mainly accumulated inside the insect cells. Interestingly, both recombinant double-fragment polypeptides A-B and B-C were secreted and could be purified to homogeneity. It was thus possible to study the properties of one recombinant fragment and two recombinant fragment pairs. All these recombinant polypeptides were folded, as judged by urea gradient gel electrophoresis and CD spectrum analysis, suggesting that they may indeed represent distinct domains.

A highly surprising finding was that the N-terminal fragment A plays no critical role in the catalytic activity of LH, because the recombinant B-C polypeptide, lacking fragment A, was a fully active hydroxylase. Furthermore, the $K_m$ values
determined for the B-C polypeptide and the full-length enzyme, including that for the peptide substrate (IKG)$_3$, were identical. Similar data have been reported previously (33) for another 2-oxoglutarate dioxygenase, aspartyl (asparaginyl) β-hydroxylase, in which a recombinant fragment lacking the 310 N-terminal amino acids possesses full catalytic activity. The catalytic subunit of the other main collagen hydroxylase, prolyl 4-hydroxylase, has separate peptide substrate-binding and catalytic domains, the former being located between residues 138 and 244 and the latter in the C-terminal region of the 517-amino acid residue human α(I) subunit (15). The present data clearly indicate that fragment A is not a peptide substrate-binding domain of LH, but they do not indicate whether the peptide-binding site is located in fragment B, which may correspond to the peptide-binding domain in the catalytic subunit of prolyl 4-hydroxylase (15), i.e. the second domain from the N-terminus.

The present data confirm the highly interesting and surprising finding (17) that LH3 also possesses collagen glucosyltransferase activity but not LH1 or LH2. The specificity of this activity has been verified by detection of the reaction product after alkaline hydrolysis (17). The level of this activity was found here to be very low, however, only about 2% of that reported for a purified collagen glucosyltransferase from chick embryos (19, 23). The $K_m$ values of LH3 for UDP-glucose and denatured citrate-soluble rat skin collagen substrate were also 4–5-fold when compared with the highest corresponding $K_m$ values reported with collagen glucosyltransferases from other sources (19). As the glucosyltransferase activity of LH3, unlike the hydroxylase activity, was not abolished by imidazole (17), the very low activity level measured here is not likely to be due to a marked degree of inactivation. The highest catalytic center activities reported for the two main collagen hydroxylases are about 400 mol/mol/min for prolyl 4-hydroxylase with the synthetic peptide (Pro-Pro-Gly)$_{10}$ as a substrate (34) and 60–100 mol/mol/min for LH1 with the peptide Ala-Arg-Gly-Ile-Lys-Gly-Ile-Arg-Gly-Phe-Ser-Gly (30, 31), both after correction for saturating concentrations of 2-oxoglutarate and the peptide substrate. The corresponding value reported for chick collagen glucosyltransferase with a saturating concentration of denatured citrate-soluble rat skin collagen as a substrate is about 45 mol/mol/min (19, 23). Exact comparison of these values was not possible, because the substrates and the in vitro reaction conditions used have differed, but the values for LH and collagen glucosyltransferase appear to be close to each other and that for prolyl 4-hydroxylase may be slightly higher. In contrast, the highest catalytic center activity measured here for the glucosyltransferase activity of LH3 is only about 1 mol/mol/min and is thus markedly lower than those of the other enzymes.

Hydroxylation of lysine residues and subsequent glycosyla-
tion of hydroxysine residues must take place in at least three
consecutive steps as follows: first, some of the lysine residues are converted to hydroxysine, second, some of the hydroxy-
sine residues are glycosylated to galactosylhydroxysine, and third, some of the galactosylhydroxysine residues are glyco-
sylated to glucosylgalactosylhydroxysine (1, 19, 35). The two
glycosyltransferase reactions have been shown to be carried out
by separate enzymes (19). The present data indicate that LH3
may also have collagen galactosyltransferase activity, but the
catalytic center activity measured for this is extremely low, less
than 0.1 mol/mol/min. As galactosyltransferase must act before
hydroxylase, it would seem virtually impossible for the LH3 polypeptide to perform all three enzymic reactions success-
sively in vivo.

All the glucosyltransferase and galactosyltransferase activity
present in the LH3 polypeptide was found to reside in

---

**Acknowledgments**—We thank Anu Myllymäki, Outi Mänty, Minna Siurua, Eeva Lehtimäki, Raija Juntunen, and Tanja Vuissinen for their expert technical assistance.

**REFERENCES**

1. Kivirikko, K. I., and Pihlajaniemi, T. (1998) *Adv. Enzymol. Related Areas Mol. Biol.* 72, 325–398
2. Myllärjä, J., and Kivirikko, K. I. (2001) *Annu. Med.* 33, 7–21
3. Nöthn, H., Nokelainen, M., Myllärjä, J., Fietzek, P. P., Müller, K. P., and Kivirikko, K. I. (1999) *J. Biol. Chem.* 274, 8989–8992
4. Norman, K. R., and Moerman, D. L. (2000) *Dev. Biol.* 227, 690–705
5. Hautala, T., Byers, M. G., Eddy, R. L., Shows, T. B., Kivirikko, K. I., and Myllärjä, R. (1992) *Genomics* 13, 62–69
6. Yeowell, H. N., Ha, V., Walker, L. C., Murad, S., and Pinnell, S. R. (1992) *J. Invest. Dermatol.* 99, 864–869
7. Valtaväärä, M., Papponen, H., Pirttilä, A.-M., Hiltunen, K., Helander, H., and Myllärjä, R. (1997) *J. Biol. Chem.* 272, 6831–6834
8. Passeja, K., Rautava, K., Kivirikko, K. I., and Kivirikko, K. I. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 10482–10486
9. Valtaväärä, M., Sipari, C., Sipari, J., and Myllärjä, R. (1998) *J. Biol. Chem.* 273, 12981–12986
10. Yeowell, H., and Walker, L. C. (1999) *Matrix Biol.* 18, 179–187
11. Pirskanen, A., Kaimio, A.-M., Myllärjä, R., and Kivirikko, K. I. (1996) *J. Biol. Chem.* 271, 8398–9402
12. Myllärjä, J., and Kivirikko, K. I. (1997) *EMBO J.* 16, 1173–1180
13. Myllärjä, K. I., and Myllärjä, J. (1998) *Matrix Biol.* 17, 357–368
14. Passeja, K., Myllärjä, J., Pirskanen, A., and Kivirikko, K. I. (1998) *FEBS Lett.* 445, 145–148
15. Myllärjä, J., and Kivirikko, K. I. (1999) *EMBO J.* 18, 306–312
16. Krol, B. J., Murad, S., Walker, L. C., Marshall, M. K., Clark, W. L., Pinnell, S. R., and Yeowell, H. N. (1996) *J. Invest. Dermatol.* 106, 11–16
17. Heikkinen, J., Risteli, M., Wang, C., Latvala, J., Rossi, M., Valtaväärä, M., and Myllärjä, R. (1996) *Biochim. Biophys. Acta* 1272, 6831–6834
18. Crossen, R., and Gruenwald, S. (1998) *Bacillus subtilis Expression Vector System, Instruction Manual, Pharmingen, San Diego, CA
19. Kivirikko, K. I. and Myllärjä, R. (1982) *Methods Enzymol.* 82, 245–304
20. Puistola, U., Turpeenniemi-Hujanen, T. M., Myllärjä, R., and Kivirikko, K. I. (1999) *Biochem. Biophys. Acta* 1411, 40–57
21. Myllärjä, R., Risteli, L., and Kivirikko, K. I. (1975) *Eur. J. Biochem.* 52, 401–410
22. Wingfield, P. T., and Pain, R. H. (1998) in *Current Protocols in Protein Science* (Wingfield, P. T., ed) pp. 7.4.1–7.4.10486
23. Antinen, H., Myllärjä, R., and Kivirikko, K. I. (1978) *Biochim. Biophys. Acta* 175, 373–742
24. Kuutti-Savolainen, E.-R. (1979) *Clin. Chim. Acta* 96, 53–59
25. Beighton, P., De Paepe, A., Steinmann, B., Tsipouras, P., and Welnstrup, R. J. (1998) *Am. J. Med. Genet.* 77, 31–37
26. Pinnell, S. R., Krane, S. M., Kenzora, J. E., and Glimcher, M. J. (1972)*N. Engl. J. Med.* 286, 1031–1036.
Lysyl Hydroxylase Fragments

27. Byers, P. H. (1995) in *Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., and Sly, W. S., eds) pp. 4029–4077, McGraw-Hill, New York.

28. Yeowell, H. N., and Walker, L. C. (2000) *Mol. Genet. Metab.* **71**, 212–224.

29. Bank, R. A., Robbins, S. P., Wijmenga, C., Breslau-Siderius, L. J., Bardoel, A. F. J., van der Sluijs, H. A., Pruijs, H. E. H., and TeKoppele, J. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 1054–1058.

30. Turpeenniemi-Hujanen, T. M., Puistola, U., and Kivirikko, K. I. (1980) *Biochem. J.* **189**, 247–253.

31. Turpeenniemi-Hujanen, T. M., Puistola, U., and Kivirikko, K. I. (1981) *Coll. Rel. Res.* **1**, 355–366.

32. Myllyla, R., Pajunen, L., and Kivirikko, K. I. (1988) *Biochem. J.* **253**, 489–496.

33. Jia, S., Mc Ginnis, K., Van Dusen, W. J., Burke, C. J., Kuo, A., Griffin, P. R., Sardana, M. K., Elliston, K. O., Stern, A. M., and Friedman P. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7227–7231.

34. Vuori, K., Pihlajaniemi, T., Marttila, M., and Kivirikko, K. I. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 7467–7470.

35. Kivirikko, K. I. (1995) in *Principles of Medical Biology* (Bittar, E. E., and Bittar, N., eds) Vol. 3 pp. 233–254, JAI Press Inc., London.
Characterization of Three Fragments That Constitute the Monomers of the Human Lysyl Hydroxylase Isoenzymes 1–3: THE 30-kDa N-TERMINAL FRAGMENT IS NOT REQUIRED FOR LYSYL HYDROXYLASE ACTIVITY
Kati Rautavuoma, Kati Takaluoma, Kaisa Passoja, Asta Pirskanen, Ari-Pekka Kvist, Kari I. Kivirikko and Johanna Myllyharju

J. Biol. Chem. 2002, 277:23084-23091. doi: 10.1074/jbc.M112077200 originally published online April 15, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112077200

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 32 references, 13 of which can be accessed free at http://www.jbc.org/content/277/25/23084.full.html#ref-list-1